Investigating the Relationship between Nitrification and Pathogen Reduction in Biological Wastewater Treatment systems

A thesis submitted for the degree of Master of Philosophy in Environmental Sciences

By

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Declaration

I, Mercedes Apeh Ugbe, hereby certify that this thesis submitted in partial fulfilment of the requirements for the award of Master of Philosophy (MPhil.) in Environmental Sciences, Abertay University, is wholly my own work unless otherwise referenced or acknowledged. This work has not been submitted for any other qualification at any other academic institution.

Signed [candidates' signature] ……MAU……...

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Supervisor's declaration:

I, Professor Joseph Akunna, hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate for the degree of MPhil in Abertay University and that the candidate is qualified to submit this thesis in application for that degree.

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I certify that this is a true and accurate version of the thesis approved by the examiners, and that all relevant ordinance regulations have been fulfilled.

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Deep appreciation to my hubby and kids who endured my stress during this time. I love you is too small.

Dedication

…To baby Jane Adriel Oluchi

ABSTRACT

This research was conducted to assess the potential of biological wastewater treatment systems in contributing to disinfection of municipal wastewater. The research focused on the microbial mediated processes occurring at secondary treatment and their effect on faecal coliform and *E. coli* numbers. The activated sludge system which is the most common biological treatment system was the case study. Focus was on the concurrent occurrence of nitrification and pathogen reduction processes at secondary treatment stage during the treatment process of municipal wastewater in which nitrogen and pathogens are already important pollutants of concern of domestic source. Specifically, the research was seeking to establish any links between both contaminants or the effects they could have on each other by assessing physico-chemical and microbial parameters.

As such, municipal wastewater was extensively aerated in lab-scale bioreactors for nitrification at secondary treatment and during this period enumeration of faecal coliforms and *E. coli* was carried out. Nitrification was observed between 4 to 11 days during which time reduction of faecal coliforms quantities was observed as well. The reduction was initially observed to be because of carbonaceous matter reduction and later by the oxidation of inorganic nitrogen which are both processes involved in wastewater nitrifying systems. Reduction of average FC numbers from 6.5 log to 4.5 log was observed during organic carbon reduction and thereafter further down to 2 log average when nitrogenous oxidation occurred. These results therefore indicated that nitrogenous oxidation was significantly negatively correlated to faecal coliform numbers (-0.907, $p=0.013<0.05$) in the thereby implying that the presence of the process of nitrification could lead to reduction of faecal coliform numbers in the system. Also, API 20E identification of persistent FC assessed and final stages of treatment process, revealed the presence of *E. coli* and FC including *Salmonella, Klebsiella, Enterobacter, Pseudomonas, Citrobacter* and *Chromo* spp. Some of which are important as pathogens as well as relevant for both biological processes.

In another experiment investigating the effects of nitrogenous oxides on faecal coliform quantities in municipal wastewater extensively aerated in the lab-scale bioreactors, it was observed that faecal coliform quantities reduced.

However, predation by protozoa was already known to be a cause of depletion of bacteria in aquatic system and therefore to confirm the observation above the effect of predation on the system was assessed. This was carried out by assessing the effects of the inhibition of protozoa activity in the system by the addition of cycloheximide, a protozoa inhibitor into the same system. Results revealed that the inhibition of protozoa resulted in an increase in the number of faecal coliforms enumerated thereby indicating that protozoan activity was a contributing factor in the reduction of faecal coliforms in this system. Further investigations revealed that the effect of protozoa on faecal coliform reduction at time of carbonaceous removal while that of nitrification was evident thereafter.

It was therefore concluded that the occurrence of nitrification in and extended aerated biological treatment system, would result in the reduction of faecal coliforms. However, further research would be required to investigate the extend and effect of this method of disinfection on other processes in the system.

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Abbreviations:

AOB Ammonia oxidising bacteria

APHA American

API 20E Analytical Profile Index

AS Activated Sludge

AS: Activated Sludge

ATP Adenosine triphosphate

BOD Biochemical Oxygen Demand

BTS Biological treatment systems

BWWT Biological Wastewater Treatment

cBOD carbonaceous Biochemical Oxygen Demand

Cfu colony forming units

COD Chemical Oxygen Demand

d Days

DNA Deoxyribonucleic acid

DNRA Dissimilative Nitrate Reduction to Ammonium

DO Dissolved Oxygen

EPA Environmental Protection Agency

EPS Extracellular polymeric Substance

F/M Food to Microorganism ratio

FA Free Ammonia

FC Faecal coliforms

h Hours

HQd High quality digital

HRT Hydraulic Retention Time

LDO Luminescence Dissolved Oxygen

MBR Membrane Reactors

Mg/L milligram per litre

nBOD nitrogenous Biological Oxygen Demand

NH_3 Ammonia

NH_3-N Ammonia Nitrogen

NH_4^+-N Ammonium Nitrogen

NO Nitric oxide

NO_2^- Nitrite ion

NO_2^--N Nitrite Nitrogen

NO_3^- Nitrate ion

NO_3^--N Nitrate Nitrogen

NOB Nitrite oxidising bacteria

PAA peracetic acid

SMP Soluble Microbial Products

Spp. species

SRT Solid Retention Time

TC Total coliforms

USEPA United states Environmental Protection Agency

UV Ultraviolet

VBNC Vulnerable but non culturable

WWTP Wastewater Treatment Plant

WWTS: wastewater treatment systems

Chapter 1. Introduction and Project Justification

This chapter introduces the study area and presents a justification for the investigation, presenting the aims and objectives considered, the conceptual framework and gives a general outline of the structure of the thesis.

1.1 General Perspective and justification

Disease carrying individuals excrete hundreds of different pathogens, which are carried along in the wastewater stream (Wen *et al.* 2009). Globally, the quantity and types of pathogens are affected by the geographical location as well as general financial status so that type of wastewater treatment systems used in location are directly influenced by these factors. However, despite the type of treatment system used and advances in water and wastewater management globally, persistent pathogens or chemicals are still found in the effluent wastewaters thereby resulting in the persistent threat of water borne diseases (Okoh *et al.* 2007) globally. This resistance to wastewater treatment methods has resulted in an increase in the emergence of waterborne pathogens (Zhuang and Jin 2003). A situation attributed to evolving food production methods, increase in population growth, increase global trade and growth in pathogen resistant to disinfections (Nwachukwu and Gerba 2004). In particular, the emergence of pathogens like *Cryptosporidium parvum* and *Escherichia coli* 0157:H7, which were responsible for the spread of cryptosporidiosis, and *E. coli* 0157:H7 infections between 2006-2009 (Luffman and Tran 2014) cast doubts on the efficiency of conventional wastewater treatment in removing pathogens.

However, several recent studies assessing the fate of pathogens in biological wastewater treatment systems, including Marin *et al.* (2015), Wen *et al.* (2009), Malham *et al.* (2014), Jimenez *et al.* (2010), Karimi *et al.* (2014), as well as Hendrick and Pool (2012) indicate the viability of these treatment plants in reducing pathogens by harnessing biological and physical factors available along line the treatment configuration. Despite this, the issues of time, land area as well as dire need for wastewater for reuse purposes like irrigation and recreation, encourages disinfection methods, which take place only at tertiary treatment stage.

Biological treatment, which harnesses the activity of microorganism, is said to be as important as chemical treatment for the reduction of pathogens in wastewater

treatment systems (Fu *et al.* 2010). This importance was attributed to the presence of sludge to which pathogens are attached in activated sludge system and the retention time, which allows physical and biological pathogen reduction processes to take place. Glass and O'Brien (1980) also noted that in biological treatment system (BTS) whose operation parameters allowed for longer aeration times, enhance pathogen removal was observed. This was because longer aeration times permitted aerobic bacteria to produce more cell biomass, which were used as settleable sludge floc unto which pathogens adhered. However, though a reduction of pathogens is observed in these treatment systems, they are not specifically designed for pathogen reduction. This implies that the pathogen reduction observed occurs by chance as a result of other processes.

As well as pathogens, raw municipal wastewater also contains nitrogen derived from nitrogenous organic matter which are also considered important pollutants especially when the receiving waterbody is abstracted for agricultural purposes or is an inland waterbody susceptible to eutrophication (Webber and Legge 2008; Marin *et al.* 2015). These are removed in biological treatment designed with long retention time after the removal of organic carbon by the process of nitrification (Burton *et al.* 2014). This process takes advantage of sludge settling and extended aeration which are also necessary for pathogen reduction (Glass and O'Brien 1980).

In the treatment of wastewater of municipal origin, pathogen and nitrogenous matter reduction are therefore two important pollutant removal processes which are likely to occur at secondary treatment if extended aeration is alllowed. As pollutants in the same wastewater stream it is possible that their interaction with one another and with other wastewater constituents would affect the occurrence or reduction of one another. More so, though some wastewater treatment plants are designed to remove nitrogen at secondary or tertiary stages, removal of pathogens usually occurs passively or is included only at tertiary stage with the use of technologies like ozonation, chlorination and UV radiation which are either expensive or have toxic by-products (Huang 2010).

Already investigation on the relevance of the interaction between wastewater contents for simultaneous removal of different pollutants is ongoing. Recent studies include the occurrence of cyanide removal processes in biological treatment of coke

wastewater (Kim *et al.* 2008a), the biodegradation of recalcitrant pollutants like artificial sweeteners (Tran *et al.* 2014) and removal of micropollutants (Yi and Harper 2007; Rattier *et al.* 2014) all in nitrifying wastewater systems. The processes in these studies all made use of long solid retention times (STR) relevant for nitrogen and pathogen removal and are microbiologically mediated. Also, Papadimitriu *et al.* (2010), showed a correlation between the removal of another municipal wastewater nutrient, phosphorus, and total coliforms in the presence of protozoa. However, though many studies have been carried out to investigate the enhancement of nitrification and pathogen removal independently in wastewater treatment systems (WWTS), up to date, none has assessed the effect these two pollutants or their removal processes could have on one another especially as both occur in same wastewater source and removal at biological treatment stage respectively.

In previous research by the way, nitrification has been referred to as an unstable process as nitrifiers are very sensitive to environmental conditions and competed for oxygen with heterotrophic bacteria (Zeng *et al.* 2014). The quantity of either group of organisms hence the stability of the process is affected by geographic location, type of treatment system and wastewater characteristics (Cydzik-Kwiatkowska and Zielińska 2016). This implies therefore that choice of treatment type and environmental conditions would predict process efficiency and stability. Previous studies also suggest that nitrification is energy intensive with respect to required aeration (Almstrand *et al.* 2011) providing oxygen, occurs after organic carbon reduction (Burton *et al.* 2014), is affected by concentrations of ammonia oxidising and nitrite oxidising bacteria (Kumari *et al.* 2011) and is influenced by the presence of bacteria predators (Pogue and Gilbride 2007). It is therefore imperative that every nitrifying treatment system would have to be adequately monitored to assess and establish stable nitrification depending on the environmental factors and constituent of wastewater being assessed.

Moreso, the instability of the process of nitrification results in the disproportionate presence of its oxide products thereby resulting in the accumulation of nitrite in the treatment system. This product and its derivatives have been observed to be toxic to bacteria in aquatic systems (Mara and Horan 2003) implying that nitrite could contribute to reduction in bacteria pathogens by its toxic action. However, though research has been carried out on its effects on other wastewater organisms like

ammonia oxidising bacteria (Kurishi *et al.* 2007) and phosphate accumulating organisms by phosphate (Saito *et al.* 2004), to date no studies have assessed its effects on the bacteria pathogens in wastewater treatment systems.

It is clear from literature therefore, that the interaction between nitrification and other contaminants is being used in wastewater treatment to enhance wastewater pollutant removal. However, though both processes of nitrification and pathogen reduction are biologically mediated and their elimination by biological wastewater treatment well researched, no studies on the relationships between these two contaminants have been published up to date. This research was therefore designed to investigate the possible effects both contaminants could have on one another.

1.1.1 Hypothesis

This research investigates the hypothesis that nitrification and pathogen reduction in biological wastewater treatment are not mutually exclusive processes.

1.1.2 Research Question

In assessing the relationship between these two removal processes, the principal question to be considered would be:

> "Is a nitrifying wastewater treatment system a disinfection one as well?"

1.1.3 Aim and objectives

The study is overall aimed at investigating the potential of nitrifying wastewater treatment systems in reducing the concentration of pathogenic organisms in biological wastewater treatment systems.

The specific objectives of this study include:

1. To identify factors affecting nitrogenous oxidation, carbonaceous removal and pathogen reduction in biological WWTS as well as identify persistent faecal coliforms therein.

2. To assess the effect of nitrogenous oxides on faecal coliforms numbers in activated sludge systems.

3. To elucidate the influence of protozoa on the processes of nitrification and faecal coliform reduction

4. To establish possible ways of system modification favourable to the enhancement of the processes of nitrification and faecal coliform reduction concurrently.

Figure 1.0 is a graphical representation of the framework which will be utilised in carrying out this investigation. It relates the research questions to the objectives and aims and links them to the different studies carried to answer them.

1.2 Organisation of Thesis

The whole study is articulated in eight chapters and this section briefly highlights the content of each. An introduction and justification of study and thesis is done here in **Chapter one**. **Chapter Two** and **Three** are literature reviews such that **Chapter Two** explores the development and occurrence of nitrification in biological treatment stage of wastewater treatment processes while **Chapter Three** evaluates pathogen reduction therein. Details of the general experimental methodology are presented and critically evaluated in **Chapter Four**. In **Chapter Five** a write up on the results and discussion of study one (figure 1.1) considering the factors affecting nitrification and pathogen removal in the lab-scale activated sludge system was carried out. **Chapter Six** presented details of methodology, results and discussion specifically relevant to study two (figure 1.1) which assesses the effects of nitrogenous oxides on pathogen concentration and the last result chapter was **Chapter Seven** which present results and discussion on the investigation which evaluates the contribution of protozoa predation to the effect of nitrification on pathogen reduction. Finally, **Chapter Eight** presents a summary of results obtained in experimental chapters Five, Six and Seven, providing a general reflection, key outcomes, conclusions and future perspectives.

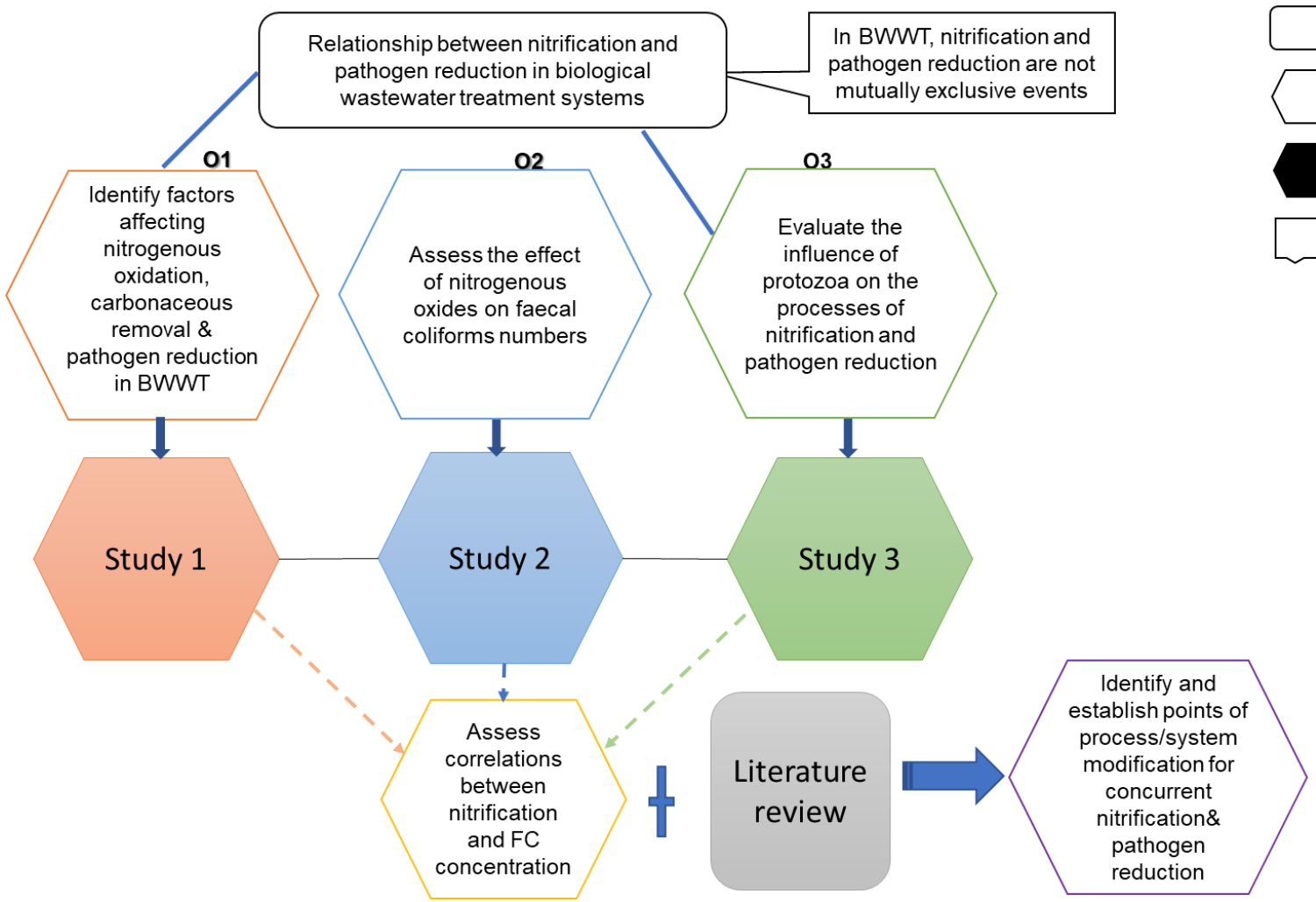


Figure 1-1 Conceptual Framework

The next two chapters are literature reviews discussing development in research on the biological processes of nitrification and subsequently, the impacts it has on pathogen reduction at secondary treatment where biological processes take place. Biological treatment in view of nitrogenous pollutants is considered and relevant organism in the wastewater stream are assessed. Other topics considered include, the factors that affect nitrification in municipal wastewater treatment plants, the effects of nitrification on the microorganisms in the wastewater stream, pathogen reduction and recent research on the effects of predation on both processes of nitrification and pathogen reduction are examined. The review is organised in two main parts: biological wastewater treatment and nitrification and pathogen reduction.

Chapter 2. Literature Review: Biological Wastewater Treatment and Nitrification

2.1 Introduction

Increases in human population and industrialization has hampered the self-purifying capacity of natural waters but has driven the development of engineered biological treatment systems whose operation mode mimic the treatment processes in natural water so that wastewater passing through them are partially or fully treated before they are discharged into receiving water bodies or reused (Pauli *et al.* 2001). This discharge of wastewater from treatment plants is considered as point source effluents which could have astounding effects on receiving waterbodies by changing hydrological patterns and nutrient processes depending on the content of the effluent discharged. However, the combined chemical, biological and physical processes employed by treatment plants are aimed at modifying wastewater content so that adverse impacts to receiving water body could be avoided; reusability improved and possibly prevent the use of advance treatment usually required for the elimination of some pollutants like nutrients and pathogens (Carey and Migliaccio 2009).

2.2 Biological Wastewater Treatment

The use of biological activity to treat wastewater has been widely applicable to wastewater of municipal and industrial sources aspiring primarily to reduce soluble organic impurities, but advance modifications have included the reduction of nutrient content to prevent oxygen depletion of receiving waters, to trap suspended solids into biofilm and in so doing to reduce the concentration of pathogenic organisms in treated effluent (Burton *et al.* 2014). The process brings active microorganisms in contact with wastewater so that soluble organics and nutrients are consumed as food (Qasim 2017). Principally, biochemical reactions performed by microorganisms in wastewater convert suspended and dissolved matter into biomass under the control of system design (Pauli *et al.* 2001). Degradation of pollutants in biological treatment could be aerobic with organisms requiring oxygen, anaerobic with organisms' not requiring oxygen or anoxic with organisms not requiring free oxygen (Schlutz 2005).

Though treatment process is identical to natural aquatic processes which reduce pollutants, characteristic such as reduce turnover time for biomass, enhanced decomposition processes, fast organic load, flow and dominance of heterotrophic bacteria indicate that they are man-made systems subjected to extreme conditions (Madoni 2011).

Biological treatment is usually at the secondary treatment stage (figure 2.1) of the wastewater treatment and is either done by suspended growth or attached growth processes (Jiménez *et al.* 2010). Typical treatment systems include activated sludge, aerated lagoons and oxidations ditches which make use of suspended growth processes while tricking filters, rotating bioreactors use attached growth processes and all these systems show different patterns of bacteria interactions.

Secondary treatment is basically designed to remove biological oxygen demand (BOD) present as dissolved organic matter not removed by sedimentation or particulate organic matter which remains in suspension and not restrained by primary treatment (Von Sperling and De Lemos Chernicharo 2017). Also, it is useful for the transformation of suspended particle into biofilms slime or floc as well as removal of nutrients, but treatment can be optimized to remove trace recalcitrant organic constituent and pathogens (Burton *et al.* 2014). Figure 2.1 shows the basic stages in municipal wastewater treatment.

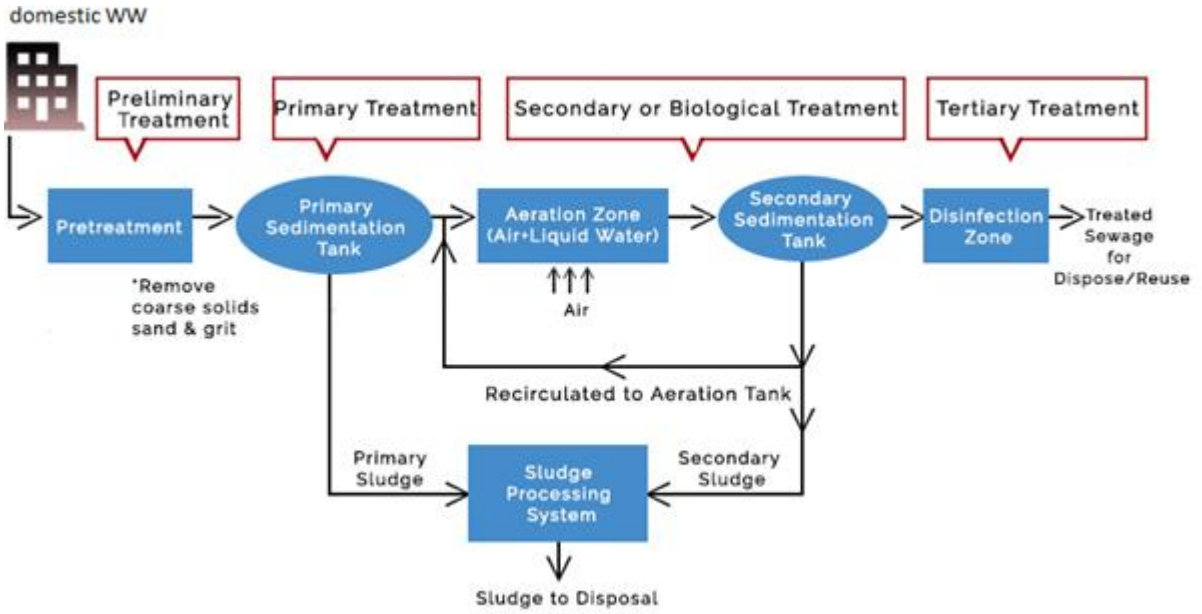


Figure 2-1 Stages in Municipal Wastewater treatment (Adapted from Akruthic Enviro Solutions 2018)

Biological treatment takes advantage of the biological metabolic processes of hydrolysis, growth and decay of microorganisms (Henze *et al.* 2001) as illustrated in figure 2.2. Organisms use simple molecules like acetic acid, glucose and ammonium and nitrite formed by hydrolysis of larger molecules in particulate and dissolve solids for growth. Hydrolysis has been observed to be the rate limiting step in BWWT as it is a slow process. As living organisms, death is inevitable and wastewater treatment exploits its occurrence as it is important in the conversion of biomass to large organic molecules (figure 2.2) which could be slowly biodegraded and adding organic substances and nutrients into the system again (Henze *et al.* 2001). The effectiveness of biological systems is dependent on cell viability which indicates the active microbial communities involved (Chouler and Di Lorenzo 2015). The relevance of viable cells in wastewater treatment is assessed by their reproductive activity which is influence by the intergrety of cell membrane and metabolic activities. Cells with intact membranes are capable of metabolic activities hence reproduction in favourable environmental conditions. (Ziglio *et al.* 2002).

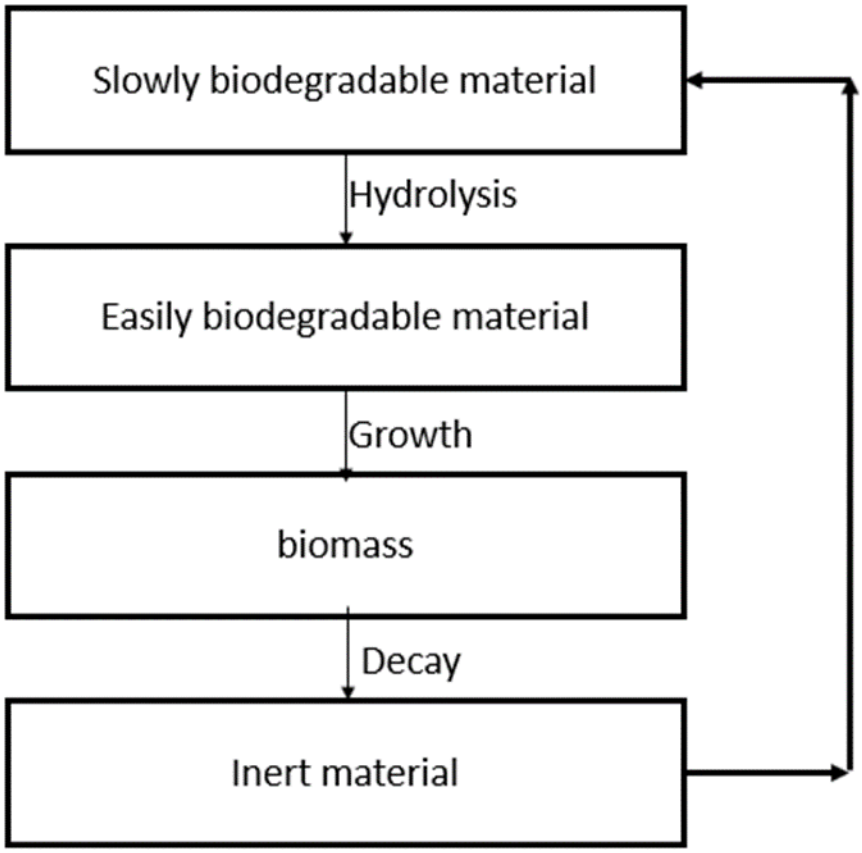


Figure 2-2 Conversions of biological material in wastewater treatment plants (Adapted from Henze *et al*. 2001).

For BWWT to take place, type of wastewater, discharge requirements, wastewater components and type of wastewater treatment system are important considerations (Shultz 2005). The wastewater biological community is transformed in the treatment plant and influenced by both the chemical and biological compositions. In some systems like those with industrial wastewater, the chemical composition is said to be of stronger influence than the biological composition (Shchegolkova *et al.* 2016). However, microorganisms in wastewater are the backbone of the biological treatment process (Henze *et al*. 2001) and the next sub sections give a review of those relevant for wastewater treatment and the purpose for biological wastewater treatment.

2.2.1 Organisms in Biological Wastewater treatment

Microorganisms in wastewater include bacteria, fungi, algae, protozoa, helminths as well as other microscopic organisms and animals that are mainly available in human faeces and aquatic environments (Burton *et al.* 2014). Minute quantities of organism could also be derived by infiltration of surface water, commercial and industrial sources into wastewater treatment system. Shchegolkova *et al.* (2016) state that a framework for the formation of a bacterial community is created by the loadings added into the mixing chamber and with time, bacteria suitable for treatment will grow. This is dependent on the influent wastewater constituent as it is supposed to be enriched with gastrointestinal tract bacteria continuously supplied with nutrients relevant for cell growth and wastewater treatment. However, influent bacteria community variations occur because of geographic place of residence and nutrition of population (Cydzik-Kwiatkowska and Zielińska 2016) but gets stable in the system over time. Henze *et al.* (2001) noted that the variety of microorganisms in any treatment plant also depends on the physical conditions predominant in the system. Their existence is enhanced by the growth medium provided by the wastewater so that competition for resources determines survival (Horan 1989) hence treatment efficiency. Wastewater treatment therefore exploits particularly bacteria, algae and eukaryotes fungi, nematodes, rotifers and protozoa whose metabolic process make use of the pollutants in the wastewater (Geraldi 2006; Burton *et al.* 2014,).

Bacteria are prokaryotes and make up the largest population of microorganisms in wastewater treatment systems (Horan 1989) and their large surface area to volume ratio as well as their metabolic and reproductive rate make them significant in wastewater treatment (Pauli *et al.* 2001). Also, present are organisms identical to bacteria in size and basic cell composition but different in cell wall and cell material, called archaea, which were only identified by molecular methods recently (Ferrera and Sanchez 2016). Archae are important in anaerobic digestion processes useful for the reduction of quantities of sludge (Burton *et al.* 2014).

The different modes of nutrition of protozoan organisms make them useful in wastewater treatment process (Horan 1989). They have been observed to exhibit autotrophic nutrition as done by the phytomastigophorea group, heterotrophic nutrition by flagellates, phagocytosis by amoeba and ciliates. Fungi feed on dead organic matter and are mainly aerobic organisms whose importance in WWTS is

seen in the proliferation of filamentous forms because of low pH (<6.5) and low nutrient availability, preventing settling of sludge in secondary clarifier (Geraldi 2006). Their ability to grow in acidic conditions as well as their ability to decompose cellulose makes them useful for composting of organic waste like sludge (Burton *et al.* 2014).

Photosynthetic eukaryotic organism present in WWTS are algae which are useful in trickling filter systems and waste stabilization ponds where they release oxygen for aerobic processes e.g. cyanobacteria (Horan 1989). Highly efficient aerobic treatment in WWTS is indicated by the presence of rotifers in effluent (Burton *et al.* 2014). In treatment systems, rotifers are observed to consume suspended bacteria and small organic particles as well as enhance floc formation (Lapinski and Tunnacliffe 2003).

Helminths and viruses have been associated with transmission of waterborne diseases (Burton *et al.* 2014). The helminth Platyhelminthes and nematodes are important in WWTS as helminth ova are indicators of faecal pollution in developing countries. These ova have been observed to show more resistance to disinfection than bacteria, protozoa or viruses (Jimenez-Cisneros 2006). Likewise, viruses which have no catabolic or anabolic functions, are also indicators of faecal contamination and are also capable of passing through treatment system (Osuolale and Okoh 2017)

Overall, municipal wastewater worldwide is more similar in composition of organic matter and nutrients than in microbial composition as differences in health conditions are reflected by differences in microorganism hence pathogen compositions of faecal origin (Jimenez *et al.* 2010). However, it is the combination of human faecal and environmental microbes that make up sewage microbial communities (Mclellan *et al*. 2010). The importance of bacteria in this study stems from the fact that bacteria pathogens constitute the most prevalent type of pathogens (Okoh *et al*. 2007) and bacteria are guilds to nitrification as well.

The next section discusses the bacteria mediated pathogen reduction processes relevant for the reduction of nitrogenous pollutants at secondary treatment. This included carbonaceous matter removal required for enough reduction of organic carbon so as to permit nitrogenous matter oxidation (Burton *et al*. 2014).

2.2.2 Soluble Carbonaceous Matter Removal

Removal of carbonaceous matter has been the primary function of biological treatment and removal of other contaminants biologically is dependent on it (Burton *et al.* 2014). After primary sedimentation carbonaceous matter mainly exist in dissolved form and can averagely be represented by the formula $C_{18}H_{19}O_9N$ (Wiesmann *et al.* 2007). Sufficient time for contact between wastewater, heterotrophic microorganisms and oxygen is necessary (Burton *et al.* 2014) for carbonaceous matter removal by oxidation of organic carbon. As microorganisms consume organic carbon as food, they carry out oxidation-reduction reactions which result in the growth of new cells and production of carbon dioxide hence increase in biomass (figure 2.3) (Grady *et al.* 2011). Heterotrophic bacteria assimilation of organic carbon is facilitated by hydrolysis of organic matter to easily biodegradable forms (equation 2.1) (Burton *et al*. 2014). The resulting new cells (biomass) (figure 2.2 & 2.3) form biological floc or biofilm when extracellular polymers are produced as a result of oxidation processes by heterotrophic bacteria. These, with specific gravity greater than that of water settle by gravity in the clarifier (Von spieling and De Lemos 2017) and are easily removed.

$$Organic\ matter\ + O_2\ + Nutrients\ \rightarrow new\ cells\ + CO_2\ + H_2O \qquad (2.1)$$

Also, by endogenous cell respiration bacteria cells are broken down to ammonia, carbon dioxide and water (equation 2.2)

$$Cells\ + O_2\ \rightarrow\ NH_3\ +\ CO_2\ +\ H_2O \qquad (2.2)$$

The rate of consumption of oxygen is directly proportional to the amount of degradable organic matter present in the wastewater at any time (Changrekar and Kharagpur ND) and removal of carbonaceous matter is hindered only when dissolved oxygen (DO) is less than 0.5mg/L.

The non-treatment of organic matter produces effluent which causes severe oxygen depletion of receiving waters as the presence of organic matter stimulates the growth of heterotrophs which require oxygen for biodegradation processes. This necessitates the estimation of the strength of organic matter in wastewater and is done in milligrams per litre of oxygen demand by either chemical or biological oxidation (Burton *et al.* 2014). Chemical oxidation with potassium dichromate

evaluates the chemical oxygen demand (COD) which is indicative of the concentration of all oxidizable organic matter in the wastewater sample (Horan 1989) whether biodegradable or not. However, breakdown of biodegradable organic matter is evaluated by assessing the biochemical oxygen demand (BOD) (Lapinski and Tunnacliffe (2003) which indicates the amount of oxygen needed by bacteria to accomplish biodegradation of organic matter. BOD could be carbonaceous as in cBOD or nitrogenous in nBOD and both are relevant when municipal wastewater is concerned (Horan 1989). COD determination is faster than BOD but is limited by the fact that it does not indicate the nature of the oxidizable matter. However, it is said to be proportional to BOD when the dissolved form of readily assimilated organic matter is being considered (Changrekar and Kharagpur ND).

The reduction of organic carbon concentration is relevant in this study to allow for growth of autotrophic bacteria which are important for the occurance of the nitrification process which is discussed in next section.

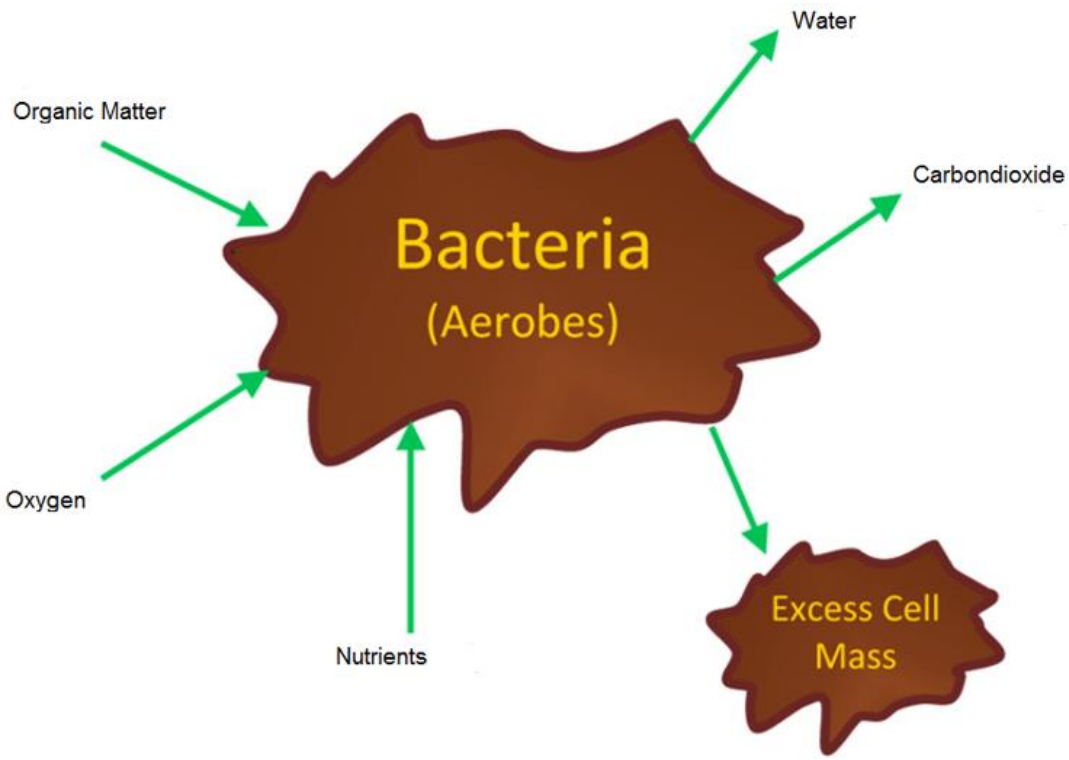


Figure 2-3 Bacteria utilization of wastewater constituents in aerobic BWWT (Adapted from Mittal 2011)

2.3 Nitrification

The complete reduction of nitrogenous pollutants is in two steps: nitrification the first and limiting step and the second step, denitrification, is dependent on it (Tanner and Kadlec 2008). As limiting step, the control of nitrification is important for efficient nitrogen removal (Pagga *et al.* 2006). This is important as nitrifying bacteria are more sensitive to change in environmental conditions than denitrifying bacteria with specific requirements for oxygen and low organic carbon as oppose to denitrifying bacteria which exist in a wider range of environmental conditions (e.g. without molecular oxygen, anoxic and high organic carbon (Kadlec and Wallace 2008).

Nitrification, the oxidation of inorganic nitrogen takes place in two stages; ammonia oxidation (nitritation) and nitrite oxidation (nitratation) which are carried out by two different types of Gram-negative bacteria, ammonia oxidising bacteria (AOB) and nitrite oxidising bacteria (NOB) respectively. They are chemosynthetic autotrophs, which are obligately aerobic for nutrition purposes (Ebeling 2000). Both types of nitrifying bacteria exist mutually in WWTS (Abeliovich 2006) and are slow growing

(Wagner *et al.* 2003). These organisms are not free standing but are present in little clusters embedded in biofilm extracellular matrix or sludge floc (Dolinsek *et al.* 2013). Nitrifiers require low organic carbon concentration (Pagga *et al.* 2006) to ensure availability of sufficient oxygen to grow. This is due to their disadvantage in competition for oxygen with heterotrophic bacteria when high organic carbon is present.

In the nitrification process, ammonia and nitrite both are electron donors used for the production of energy while inorganic carbon (HCO_3^-) is source of carbon for growth (equation 2.3) (Horan 1989).

$$NH_4^+ + O_2 + HCO_3^- \rightarrow C_5H_7O_2N + NO_3^- + H_2O + CO_2 \qquad (2.3)$$

Nitrification consumes 7.14 g of alkalinity and 4.57 g of dissolved oxygen for every gram of ammonia nitrogen catabolized to nitrate nitrogen hence a need for continuous supply of oxygen into a nitrifying system and as well as pH monitoring to identify and prevent acidic conditions (Henze *et al.* 2001). The growth rate of nitrifying bacteria is important for the nitrifying process to proceed and this is influence by both physicochemical and biological parameters such as substrate availability, dissolved oxygen concentration, pH, light, temperature and microbial community composition (EPA 2002).

Of the two nitrification steps, nitritation, the production of nitrite, is the rate limiting one (Kowalchuk and Stephens 2001) as AOB are more sensitive to environmental change and are of slower growth rate such that as starters of the process, the unpredictability of their occurrence has made nitrification to be regarded as an uncertain process (Bellucci *et al.* 2011). However, the relationship between AOB and NOB in a nitrifying wastewater system is said to be of high mutual benefit as NOB gets electron donor from AOB while AOB depend on NOB to detoxify the system by using up NO_2^- (Rittmann & McCarty 2003). Conversely, this relationship is described as chaotic and fragile due to limited specie variety of both guilds thereby, contributing to the instability of the process (Graham *et al.* 2007). The ammonia and nitrite concentrations, carbon and nitrogen ratio, solid retention time and the presence of inhibitory substances have been shown to affect the rate of nitrification (Tang and Chen 2015). There is heterogeneity in nitrification rates in aquatic environments because of the relative abundance of nitrite and ammonia oxidisers in

systems due to the physiochemical properties mentioned above as well as wastewater composition and seasonal variations (Kumari *et al.* 2011). Different nitrification rates were also attributed to system design e.g. systems with low hydraulic retention resulted in biomass washout but favoured the growth of dominant nitrifiers: *Nitrosomonas* and *Nitrobacter* species (Almstrand 2012).

Also, nitrifiers are classified as k- or r-strategist depending on DO and ammonia concentrations (Burton *et al.* 2014) such that at low substrate concentrations k-strategist grow faster while in high substrate concentrations r-strategist grow faster (Limpiyakorn *et al.* 2007). Amongst the AOB bacteria *Nitrosospira* are the k-strategist while *Nitrosomonas* are the r-strategist. So that depending on substrate concentrations population shift occur in favour of the most favourable specie e.g. the predominance of AOB in laboratory reactor when DO concentrations changed from 8.5 mg/L to 0.24 mg/L (Park and Noguera 2004). However, AOB have been observed to have a high affinity for oxygen then NOB and predominate in low oxygen environments (Tanner 2004). These therefore explaining the variability of nitrifyers which would influence stability of nitrification

Recently however, research has reported the involvement of some nitrifying organisms in minute denitrifying activity. This was observed to be due to the increase in antropogenic nitrogen supply to the environment (Shailaja *et al.* 2006; Klotz and Stein 2008) which caused high oxygen depletion of water bodies so that denitrification by nitrifyers occured. However, some *Nitrospira e.g Candidatus nitrospira defluvii,* said to be the most abundant NOB in wastewater treatment have been observed to denitrify in oxygen limiting conditions due to possession of the nitrite reductase genes (Lucker *et al.* 2010) which enable producton of nitric oxide.

Contrary to the importance of nitrification in the WWTP, nitrate formation is sometimes deleterious, as nitrates have been released to ground water in the process, thereby contaminating portable water sources or as a result of low pH, prompting the release of metals which could be toxic, into the water system (Zhang *et al.* 2010). A brief description of the the stages of nitrification and factors affecting the occurance of nitrification will be discussed in the preceeding sections.

2.3.1 Ammonia oxidation

Nitrosomonas, *Nitrosospira* and *Nitrosococcus* are ammonia oxidising bacteria (AOB) identified as responsible for nitritation (equation 2.4 and 2.5). These obtain energy by oxidation of ammonia, converting it to nitrite (Almstrand 2012). Ammonia exist in water in two forms: free ammonia and solvated as ammonium ion both existing in equilibrium highly influence by pH such that in high pH free ammonia is predominant but NH_4^+ is predominant at neutral pH (Nollet and Gelder 2000).

$$NH_3 + O_2 + 2H^+ + 2e \rightarrow NH_2OH + H_2O \quad (2.3)$$

$$NH_2OH + H_2O \rightarrow NO_2^- + 5H^+ + 4e \quad (2.4)$$

The reaction initially results to an intermediary compound hydroxylamine (equation 2.3) (Prosser 1986) by ammonia monooxygenase enzyme in an endergonic reaction. The resulting hydroxylamine is then converted to nitrite by hydroxylamine oxidoreductase with the help of oxygen in water in an exergonic reaction (equation 2.4) which also causes acidity as a result of production of hydrogen ions (Prosser 1986). Though ammonium is the energy source for AOB, not all of it in solution is used for nitrification as some is used for growth of new cells using carbon dioxide as carbon source (equation 2.5) (Ge *et al.* 2010, Horan 1989).

$$NH_4^+ + O_2 + HCO_3^- \rightarrow C_5H_7O_2N + NO_3^- + H_2O + CO_2 \quad (2.5)$$

The ammonia monooxygenase enzyme involved in ammonia oxidation is said to be a non-specific enzyme and its presence has been seen to enhance other biological processes e.g. the biodegradation of wastewater pollutants like artificial sweeteners (Tran *et al.* 2014) and micropollutants (Rattier *et al.* 2014; Yi and Harper 2007) thus adding to the importance of nitrification in wastewater treatment systems.

AOB are of β and γ proteobacteria types but those involved in wastewater treatment mostly belong to the β-proteobacteria of the *Nitrosomonas* genus (Koops *et al.* 2006). Substrate affinity (Yuchi *et al.* 2011), salinity (Bollmann *et al.* 2002), nitrite concentration (Yu and Chadran 2010), oxygen (Belluchi *et al.* 2011) as well as pH & temperature (Koops *et al.* 2006) have been shown to affect the presence of AOB and this is further discussed in section 2.2.4.

Previous research suggests the presence of both anaerobic and aerobic ammonia oxidation as some ammonia oxidisers show versatilty in metabolism thus capable of

denitrifying autotrophically in different situations e.g. when oxygen is limited, under anoxic conditions with organic carbon or hydrogen or even using N_2O_4 during both oxic or anoxic conditions. *N. eutropea* is an anaerobic ammonia oxidiser at very low oxygen concentration in biofilms formed on soil particulate material in the presence of nitrite producing hydroxylamine and nitrite (Prosser 1986). Also, molecular studies identified ammonia oxidising archaea as other contributors to ammonia oxidation in WWTS (Zhang *et al.* 2010; Limpiyakorn *et al.* 2011). However, these other processes are said to contribute minimally to ammonia oxidation (Mußmann *et al.* 2011).

2.3.2 Nitrite Oxidation

Nitrite oxidising bacteria (NOB) *Nitrobacter*, *Nitrospina*, *Nitrococcus*, and *Nitrospira* have been most commonly observed as responsible for the second step of nitrification, nitratation, (equation 2.6) (Prosser 1986). They obtain energy from the oxidation of nitrite to nitrate (Ge *et al.* 2010).

$$NO_2^- + H_2O \rightarrow NO_3^- + 2H^+ + 2e \qquad (2.6)$$

The reaction is catalysed by the enzyme nitrite oxidoreductase and *Nitrobacter* (α-proteobacteria) as well as *Nitrospira* (δ- proteobacteria) are said to be the dominant NOB in WWTS (Ge *et al.* 2015). *Nitrospira* is usually abundant in conditions of high inorganic carbon while *Nitrobacter* is available in condition of low inorganic carbon (Fukushima *et al.* 2013). Also, *Nitrospira* is said to have high substrate affinity and low maximum specific growth rate (k-strategist) while *Nitrobacter* was identified under limited substrate condition (r-strategist) (Hwang *et al.* 2010). The mixotrophic activity of some NOB as discussed in section 2.3 results in the prevalence in numbers of nitrite oxidisers to ammonia oxidisers in aquatic systems (Prosser 1986).

2.3.3 Heterotrophic nitrification

This process is of significantly lower rate than autotrophic nitrification but has been identified in wastewater systems (Grady *et al.* 2011). It results from the oxidation of inorganic or organic forms of nitrogen to nitrate by fungi and heterotrophic bacteria. In some organisms like *Bacillus sp*. LY (Zhao and He 2009), *Pseudomonas stutzeri, Paracoccus denitrificans* (Su *et al.* 2001), this is associated to aerobic denitrification and in fungi to lignin degradation (Prosser 1986).

2.3.4 Factors affecting nitrification

Dependence on energy and metabolic activity makes nitrification to be linked to growth rates of nitrifying organisms (Juliastuti *et al.* 2003). The growth and development of nitrifiers hence nitrification is affected by environmental as well as system design factors which will be discussed in this section.

2.3.4.1 pH

The acidity of aqueous media is represented numerically in pH measurements (Mesmer and Holmes 1992) and it influences the rate of utilization of metabolic substrates as well as activity of microbial organisms thereby influencing sludge formation and pollutant removal (Yan *et al.* 2013). In WWTS carbon dioxide stripping possibly due to aeration vibration processes and oxidation of ammonium nitrogen (NH_4^+-N) have been observed to cause changes in pH (Ge *et al.* 2015). pH is said to strongly affect nitrification and previous research shows pH range for nitrification to be between 6-9 (Chen *et al.* 2006) while optimal nitrification activity was noted at pH of 7.5 to 8.5 (Coskuner and Jassim 2008; Campos *et al.* 2007). Reduced nitrification observed below pH of 6.5 was attributed to reduction in ammonia species which are the substrate. pH of 7 to 8 has been observed as optimum for *Nitrosomonas* while 7.2 to 8.1 for *Nitrobacter* but adaptation of bacteria to treatment system conditions have led to the wide range of pH mention above (Ebeling 2000). However, rapid changes in pH will stress bacteria and lower nitrification activity. The effect of pH on nitrification is observed in changes in free ammonia and nitrous acid concentrations as well as affecting reaction rates of biological processes (Campos *et al.* 2007). Free ammonia is said to inhibit both NOB and AOB at concentrations of 0.1 to1 mg/L and 10 to100 mg/L respectively (Anthonisen *et al.* 1976; Kim *et al.* 2008b). Ammonia in solution, as a result of ammonification, is in the form of ammonium ion (NH_4^+) and high pH favours NH_3 whose presence in turn favours the nitrification process (Anthonisen *et al.* 1976). Also, as nitrification proceeds the formation of nitrite which exist in equilibrium with nitrous acid (HNO_2) results in the release of H^+ causing a decrease in pH hence a negative feedback on the nitrification process itself.

2.3.4.2 Alkalinity

The process by which water neutralises acid or absorbs hydrogen ions is called alkalinity (Marietta 2015) and is a measure of the amount of base that neutralise acids in water. Nitrification is an acid forming process hence its progress means a

loss of alkalinity in the system affecting the pH (Henze *et al.* 2001). The consumption of alkalinity is said to be the inorganic carbon source of heterotrophic nitrifying bacteria and also helps to balance the level of acid to bases in the mixture hence affecting the state of matter in aqueous solution (Hou *et al.* 2014). Wastewater biological entities are optimally active between pH of 7 to 8, which is alkaline (Marietta 2015). Henze *et al.* (2001) state that 7.14 g of alkalinity as calcium carbonate is lost per gram of ammonia nitrogen catabolized during nitrification (equation 2.7)

$$NH_4^+ + 1.83O_2 + 1.98HCO_3^- \rightarrow 0.021C_5H_7O_2N + 0.98NO_3^- + 1.041H_2O + 1.88H_2CO_3^- \quad (2.7)$$

A reduction in alkalinity is caused by an increase in acidity which eventually leads to reduction in nitrification rate when pH gets below 6. Common buffers used include sodium bicarbonate or sodium hydroxide (Ebeling 2000). This control of alkalinity is particularly important during nitrification of soft water where the pH is at times too low for nitrification (Henze *et al.* 2001).

2.3.4.3 Temperature

Nitrification activity has been observed at temperatures between 8 to 50°C however 25-30°C was identified as range for optimal growth of most nitrifying bacterial strains and optimal nitrification activity has been observed at 30 to 38°C (Coskuner and Jassim 2008). However, other authors believe that at temperatures above 25°C there exists differential growth between AOB and NOB bacteria as a result of higher growth rates for AOB than for NOB (Ge *et al.* 2015). This implies that treatment plant configurations which allow for low temperature like reduced hydraulic retention time, reduce nitrifying bacteria activity (EPA 2004) hence less nitrification rates. Temperatures below 10°C and above 50°C cause a reduction in maximum specific growth rate of microorganisms and affect enzyme activity hence preventing metabolic activities like nitrification (Coskuner and Jassim 2008). For example, in an anaerobic ammonium oxidation (ANNAMOX) process, ammonia oxidation was observed at 40°C while nitrite oxidation was prevented (Van Dongen *et al.* 2001). An evidence of the two oxidisers operating under different maximal temperatures.

The effect of temperature on nitrification is more evident in suspended growths system than in the fixed film types according to previous research. Zhu *et al.* (2007)

proved that between temperatures of 14° to 27°C in fixed film systems no significant impact on nitrification was observed hence the predicted impact of temperature on nitrification in biological systems is exaggerated.

2.3.4.4 Oxygen concentration

Useful as dissolved oxygen (DO) in water, nitrification is said to account for up to 40% of mixed liquor total oxygen demand (Hong *et al.* 2012). Wang *et al.* (2014) identified it a vital variable which influenced the structure of microbial community of AOB in full scale and laboratory scale bioreactors. For every gram of ammonia nitrogen catabolized 4.57g of oxygen are required (Bioscience 2014). DO levels in mixed liquor are not available to nitrifiers till they can penetrate nitrifying floc (Princic *et al* 1998) and researchers (Coskuner and Jassim 2008; Malone 1998; Wheaton 1995) have recommended DO of 2 mg/L minimum in influent for onset of nitrification activity. Oxygen affinity constants of 0.36 mg/L for ammonium oxidisers and 1.1 mg/L for nitrite oxidisers are recorded in literature, hence nitrite oxidisers are more sensitive to oxygen limitations and sometimes oxygen is responsible for nitrite accumulations in WWTS (Campos *et al.* 2007). Specifically, AOB like *Nitrosomonas* were observed to require DO of 2.0 mg/L minimum while NOB like *Nitrobacter* required at least 4.0 mg/L for growth (Ebeling 2000). Moreso, nitrification was inhibited at DO concentration of 0.3 mg/L (Pogue and Gilbride 2007).

2.3.4.5 Salinity

This is particularly relevant in parts of the world where fresh water sources are limited, hence seawater is being used in domestic activities like flushing (Wu *et al.* 2008). The percolation of saline water into sewerage system as well as tanning and cheese manufacturing industries are sources of increase salinity into municipal wastewater. Salinity is usually a measure of the chloride content of wastewater (Heitmann 1990) and if given sufficient time, nitrifying bacteria can adapt to changes in dissolve salt concentration. However, abrupt changes of about 5 g/L will cause a shock to nitrifying bacteria hence nitrite nitrogen and ammonia nitrogen reaction rates will be decreased (Peng and Zhu 2006). The common occurrence of chloride salts in domestic wastewater hampers biological treatment, as high salts concentrations are responsible for high osmotic concentration and low dissolved oxygen (Tian *et al.* 2014). These factors have been seen to cause reduction in

abundance and activity of ammonia oxidising bacteria (Yang *et al.* 2011) hence limiting the process of nitrification.

2.3.4.6 Light

In bio-media systems light has resulted in increased growth of algae but a reduction in growth of nitrifying bacteria (Ebeling 2000) thus a reduction in nitrifying activity. Earlier research shows that nitrifiers are sensitive to florescent and visual light as well as UV light resulting in reduced activity, but nitrification has been observed in dark conditions (Wolfe *et al.* 2001). Much more, in shaded areas excision repair of DNA is stimulated in nitrifiers hence there is recovery of nitrifiers in shaded environments (Wolfe *et al.* 2001).

2.3.4.7 Substrate concentration

The concentration of ammonia nitrogen which is substrate results in a direct increase in rate of nitrification to at least 30 mg/L (Ebeling 2000) but rate reduces thereafter, and extremely high concentrations of ammonia and nitrite have been shown to inhibit nitrification totally. This is due to the formation of free ammonia at high concentrations of ammonium (Anthonisien *et al.* 1976). However, at 350 mg/L of ammonia nitrogen no toxic effects were observed on nitrifiers in a full-scale nitrifying system (Kim *et al.* 2008).

Both types of nitrifying bacteria exhibit sensitivity to substrate concentration differently such that at low ammonium concentrations (<10 mg/L) AOB are more abundant than NOB (Princic *et al.* 1998). Earlier research also stated that NOB require three times much more nitrogenous substrate than AOB (Verstraete & Focht 1977) hence a need for substantial amount of ammonia to support the process.

Relevant to the effect of substrate on nitrification are suspended solids. Xia *et al.* (2004) stated that suspended solids present in water enhanced nitrification non – linearly even at low concentrations of 1 mg/L of ammonium nitrogen. They explained that the presence of suspended solids increased the likelihood of contact between nitrogen and bacteria, as well as make available nutrients necessary for nitrification.

2.3.4.8 Treatment Operating Conditions

Different types of treatment systems as well as operations conditions affect the way in which substrates are utilised hence the nitrification process. Ammonia loadings, organic loading and sludge age will be considered here.

2.3.4.8.1 Ammonium Loading

Nitrification is enhanced in systems in which nitrifiers are not starved of ammonium for long periods (Wilk 2000). This occurs in plug flow activated sludge or trickling filter systems where a gradient of ammonium concentration is formed as nitrification takes place so that eventually nitrifiers are starved and die leading to nets decrease in nitrification especially at peak loads. Nitrification potential is therefore dependent on the system's ability to deal with different loadings of ammonium (Almstrand *et al.* 2011). In the Rya WWtp (Gothenburg) alternating ammonium loads between high and low were more successful in maintaining nitrification in the system than constant loading (Almstrand *et al.* 2011). Another strategy was to switch the order of nitrifying tricking filters thereby alternating ammonium loading rates causing nitrification rate to be higher than with continuous or intermediate concentration imputes (Andersson *et al.* 1994). More recent laboratory experiment showed that for specific AOB bacteria populations (Bollman *et al.* 2002) as well as industrial wastewater, nitrification activity could be sustained by starving system for 15-36 days (Almstrand *et al.* 2011).

2.3.4.8.2 Organic loading

The organic loading influences the food to microorganism (F/M) ratio. This ratio relates substrate utilization to biomass generation thereby indicating the ability of biological process occurrence. For extended aeration and complete mixed reactors used for nitrification in activated sludge systems its value should be between 0.05 to 0.15 and 0.2 to 1.0 respectively (Mishoe 1999).

At secondary treatment in aeration tanks nitrification is affected by organic loading expressed as BOD_5. It is said to be the only obligate wastewater parameter, which could be related to microbial characteristic of wastewater treatment (Muela *et al.* 2011). Organic matter promotes the growth of heterotrophs capable of outcompeting nitrifiers for dissolve oxygen so that heterotrophic activity prevents nitrification except in situations where oxygen levels are in excess (Tchobanoglous *et al.* 2003). This implies that influent wastewater with relatively high organic load will cause an

increase in the growth rate of heterotrophs in the presence of oxygen and therefore organic load needs to reduce considerably before the onset of nitrification. Also, extensive presence of soluble microbial products (SMP) in nitrifying bioreactors have been observed as another source of organic matter in the system (Rittmann *et al.* 1994). These are released by ammonia and nitrite oxidising bacteria as well as during the decay nitrifying biomass (Dolinsek *et al.* 2013) and their presence has been observed to support heterotrophic organism's growth in WWTP hence limiting nitrification.

2.3.4.8.3 Sludge age

The nitrifying ability of an activated sludge system is dependent on the mean cell residence time (MCRT), solid retention time (SRT) (Tang and Cheng 2015) or sludge age. This is a measure of the average time the sludge stays in the biological reactor (Bozek *et al.* 2005). If the system's sludge age is lower than nitrifier generation time, wasting of excess activated sludge will result in nitrifiers being washed out before their growth rate hence impairment of nitrification (Wang *et al.* 2010). Sludge load which is a ratio of removable substrate to a kilogram of dehydrated activated sludge per day usually measured in terms of BOD_5, also has an influence on the sludge age as well as the retention time of wastewater (See table 2) in the biological reactor (Bozek *et al.* 2005).

However, the biomass content of biological reactor increases with increase in SRT as a result of growth, but a saturation point is attained where increase is stationary then the biomass becomes inert. This reflects also in the ammonia oxidation and nitrite oxidation which get to a stationary point as well (Burton *et al.* 2014).

Table 1 Relationship between sludge loading, sludge age and retention time approximately (Adapted from Bozek et al. 2005)

Load	**Sludge age (d)**	**Sludge load according to BOD_5, (kg/kgd)**	**Retention time of WW Biological reactor (h)**
Low load	>25	0.05-0.1	24-72
Medium load	3-15	0.2-0.5	4-12
High load	<3	>1	1-2

Nitrifying organisms have different specific growth rates implying that sludge age will affect their concentration differentially (Yuan *et al.* 2008). In fact, Peng and Zhu (2006) found a shorter doubling time for AOB than for NOB in their work. However, SRT of 10-30 days with extended aeration has been observed in nitrifying systems (Tchobanoglous *et al.* 2003; Campos *et al.* 2007) to allow for growth of sufficient bacteria especially as nitrifiers are slow growers (Coskuner and Jassim 2008). This sludge age is longer in winter due to low temperatures lowering bacterial activity but shorter in summer (Li *et al.* 2015). Bioaugmentation of the biological reactor in a nitrifying system with selected strains of nitrifying bacteria which enhances growth of endogenous nitrifying population is recommended to reduce sludge age requirement and limit impairment of nitrification as a result of sludge wasting (Head and Oleszkiewics 2004; Tang and Chen 2015) in system upgrades or in very cold climates. In another research, Pollice *et al.* (2002) reported a reduction in the oxidation rate of ammonium oxidisers to 14% when SRT was increased from 3 to 24 days at 32°C implying that there is possibly a maximum point to which sludge should be retained. SRT is dependent on temperature e.g. at 18 to 25°C BOD removal takes 3 days and 4 to11 days for nitrification (Burton *et al.* 2014).

Posso-Blandon (2005) suggested the addition of carbon dioxide into nitrifying reactors to reduce SRT as inorganic carbon is important in the synthesis of nitrifying cell (equation 2.7) thereby enhance nitrification. Moreso when inorganic was treated in a conventional activated sludge system, low hydraulic retention time resulted in biomass washout but favoured the growth of dominant *Nitrosomonas* and *Nitrobacter* specie which continued nitrification in the system (Kumari *et al.* 2011). However, low HRT will not favour pathogen removal especially as one of the routes of removal is sedimentation.

2.4 Biological Wastewater nitrifying systems

Secondary treatment, where biological nitrification occurs, is achieved by either suspended growth or attach growth processes. In the latter bacteria are kept in suspension by mixing to obtain complete mixing of the system while in the former the bacteria are attached as slime on a surface (EPA 2014). Table 2.2 shows nitrifying systems and different configurations they present. It indicates that nitrification is always preceded by organic carbon reduction. Most wastewater sources containing

ammonium also contain organic matter so that carbon oxidation takes place in the same system where inorganic nitrogen oxidation takes place (Henze *et al.* 2001). This implies that any nitrifying system should have facilities for both processes.

The activated sludge system generally operates the suspended growth process which mimics the self-purification biological process of suspended bacteria in rivers and streams. However, the fixed film system like trickling filter, mimics the slime growth of receiving waters. In this section different configurations of the activated sludge, the most commonly used system for biological treatment, will be explored and a brief discussion on the trickling filter system will be done.

Table 2 Wastewater treatment systems capable of nitrification (Adapted from Burton *et al*. 2014)

Type	Common name	Use
Suspended growth	Activated sludge	cBOD & nitrification
	Aerobic Lagoons	cBOD & nitrification
	Membrane bioreactor	cBOD & nitrification
Attach growth	Biological aerated filters	cBOD & nitrification
	Moving bed bioreactors	cBOD & nitrification
	Rotating biological contactors	cBOD & nitrification
	Trickling filters	cBOD & nitrification
Hybrid Processes	Trickling filter/AS	cBOD & nitrification
	Integrated fixed film AS	cBOD & nitrification
Combined aerobic, anoxic and anaerobic processes		cBOD, nitrification, denitrification & phosphorus removal
Lagoon Processes	Aerobic lagoons	cBOD & nitrification
	Maturation lagoons	cBOD & nitrification

2.4.1 Biofilms

Important for biological degradation of pollutants are microbial biofilms, which occur either as slime in attached growth processes, or floc in suspended growth processes

of WWTS. These occur as a result of the spontaneous accumulation of dissolved organic molecules on surfaces of different materials creating a conditioning film on which bacteria cells get immobilized and produce extra polymeric substances unto which cells get attached and from which (Costerton *et al*. 1995). An equilibrium exists between cells attached and those in suspension and this is dependent on the prevailing ecological and physico-chemical conditions as well as design characteristics of treatment system involved (Pauli *et al*. 2001).

2.4.2 Suspended growth processes - Activated sludge (AS)

As mentioned above there are different types of suspended growth processes but here the AS system, which is most commonly used and widely studied is considered. In the AS system, primary effluent wastewater is mixed with microorganisms in suspension in the presence of oxygen with an aim to stabilize organic matter (Qasim 2017).

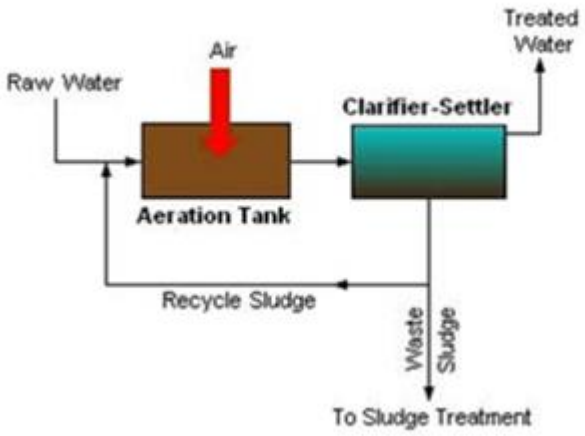


Figure 2-4: A sketch of the activated sludge system (Adapted from Fuller 2018)

This mixed community comprises bacteria, protozoa, fungi, rotifers and sometimes nematodes but constant agitation of system for aeration limits growth of higher organisms (Hagen 2018). The system consists of a biological reactor in which organism are growing in controlled conditions connected to a clarifier. Primary effluent gets into the biological reactor where it is aerated, mixed and combined with returned activated sludge. Organic matter is converted here into new cells partly, carbon dioxide and water to derive energy. New cell form clumps by interactions in the system and the mixture called mixed liquor is moved to the separation unit where

sludge is separated from effluent wastewater (Bozek *et al.* 2005). Settled sludge is move back to aeration reactor to maintain a proper food to microorganism ratio important for effective biodegradation (Qasim 2017). Also, excess sludge is sent for sludge management as waste activated sludge.

Usually the AS system comes after primary treatment, but this is not so in hot environments where primary wastewater treatment could produce odour problems. Here, use of other versions of AS such as oxidation ditch, membrane reactors or sequence batch reactors is employed (Burton *et al.* 2014).

Important for the supply of oxygen is introduction of air into the aeration tank. This is achieved by mechanical or diffused means. In diffused aeration air is pumped in by use of porous diffusers or air nozzles at bottom or side of aeration tank while with mechanical aeration air is put into the treatment system from the atmosphere mechanically by surface impellers or submerge turbines e.g. used in aerated lagoons or aeration basins (Qasim 2017).

Different configuration of AS include:

2.4.2.1 Plug flow

Here, the composition of wastewater varies along the reactor as it flows along the aeration chamber. This variation is due to differences in sludge and oxygen concentrations as no mixing takes place (Burton *et al.* 2014). However, it is assumed that mixing occurs cross sectionally. Oxygen concentration decreases from influent to effluent and bacteria concentration decreases from influent to effluent (Wiesmann *et al.* 2007). They have shown rapid onset of nitrification as well as produce good settleable sludge (Azim and Horan 1991) because of increased substrate concentration at influent. However, due to back mixing caused by aeration, pure plug reactor is difficult to get and selection as well as operation of this system is difficult (Horan 1989).

A modification of this system is seen in step feed process. Here, primary effluent is put in at several points in aeration tank hence equalising F/M ratio so that peak oxygen demand is reduced (Burton *et al.* 2014). This method of input of wastewater allows for flexibility in operation but can only nitrify partially due to short SRT

2.4.2.2 Completely mixed reactors

Primary effluent is completely mixed with sludge and air so that composition of wastewater at any point of aeration chamber is similar. Here aeration chamber is circular or square (Qasim 2014) and mixing of wastewater dilutes concentration of primary effluent thereby reducing the effect of any toxicants to microorganisms in the system as well as enhances contact with oxygen. There is therefore uniformity in oxygen demand so that it is easy for engineers to design reactors with right size and operation parameters to meet oxygen requirements (Horan 1989). However, Azim and Horan (1991) found that nitrification rates here were slow and sludge settling was poor because of the availability of free ammonia.

2.4.2.3 Sequence batch reactors

Here all stages of activated sludge process including filling, aeration, sedimentation, draw and idle, take place sequentially in one or more tanks reactor and no sedimentation tank is required (Burton *et al.* 2014). This single completely mixed reactor is quite useful when variable organic and hydraulic loads as well as limited skill and operator controls are available (Horan 1989).

2.4.2.4 Contact stabilisation

Here the conventional AS is modified so that the aeration tank has two compartments: one for contact of primary effluent and stabilized sludge and the other for stabilization. It is characterised by short SRT hence limited nitrification (Horan 1989).

2.4.2.5 Extended Aeration

An AS system with an extended aeration tank into which unsettled primary effluent is put and aerated and mixed for long SRT of about 20-30 days. Long SRT provides excellent condition for nitrification (Burton *et al.* 2014). This configuration is applicable in systems like the Biolac, Counter Current Aeration System and the Cyclic Activated Sludge System.

The oxidation ditch also displays extended aeration in an oval tank, but wastewater flow mimics the plug flow system as mechanical aeration provides mixing unidirectionally. Its design has been improved in the Orbal systems which employs concentric channels for mixing mixed liquor but like in oxidation ditch oxygen becomes limited toward end of channel permitting denitrification (Burton *et al.* 2014).

2.4.2.6 Membrane Bioreactors (MBR)

These are conventional activated sludge biological reactors into which micro or ultrafiltration membranes are added so that the membrane acts as a barrier to solids in suspension and microorganisms (Chae *et al.* 2014). Significant removals of human enteric and coliform bacteria have been observed in full scale MBR (Xagoraraki *et al.* 2014) but removal of viruses has not been significant due to small sizes (Herath *et al.* 1999). Cyst and oocyst of protozoan organism are 3-12µm which is larger than pore size of filters in MBR hence complete removal is expected. However, the small sizes of virus have made their reduction with this system questionable (Hai *et al.* 2014). MBR are said to remove pathogens to a comparative level like a conventional activated sludge system plus their tertiary treatment (Ottoson *et al.* 2006) therefore it's a very efficient design for pathogen reduction. In the operation of nitrifying activated sludge systems, the configuration of the aeration tank and the formation of biofilms are important operation parameters.

2.4.2.7 Configurations of AS completely mixed systems

Nitrifying wastewater systems are configured to either carry out both processes of organic carbon reduction and nitrification in one aeration tank in a single stage system whereby both processes occur in same biological reactor or in two aeration tanks in a two-stage system such that carbonaceous matter oxidation take place before nitrification (Burton *et al.* 2014) as represented in figure 2.5 and 2.6 respectively.

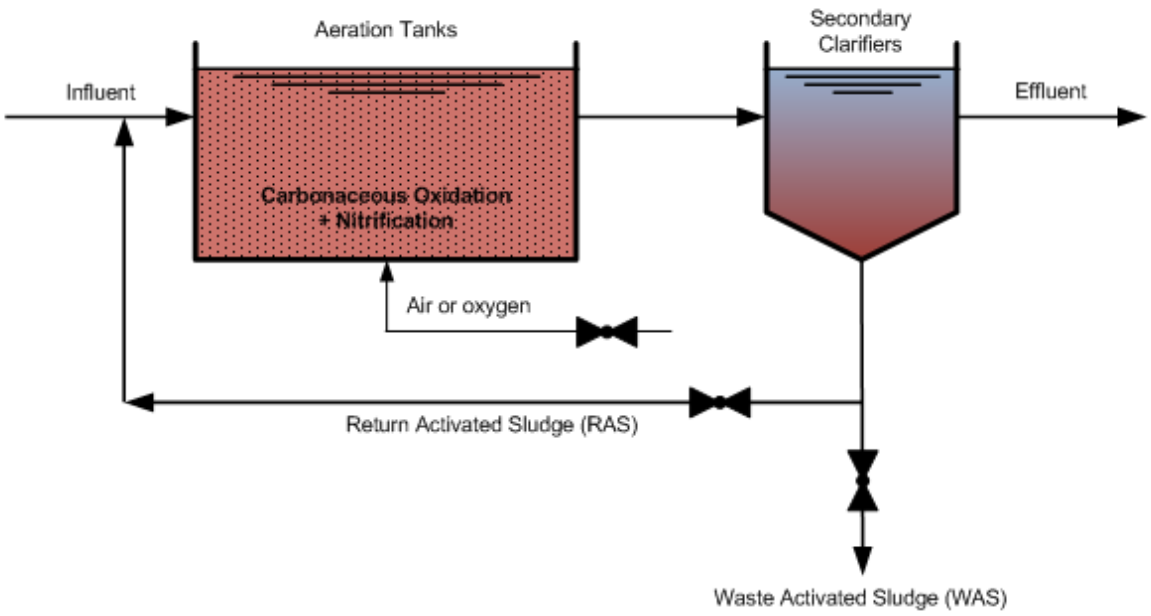


Figure 2-5: AS system with single stage nitrification (Adapted from Fuller 2017)

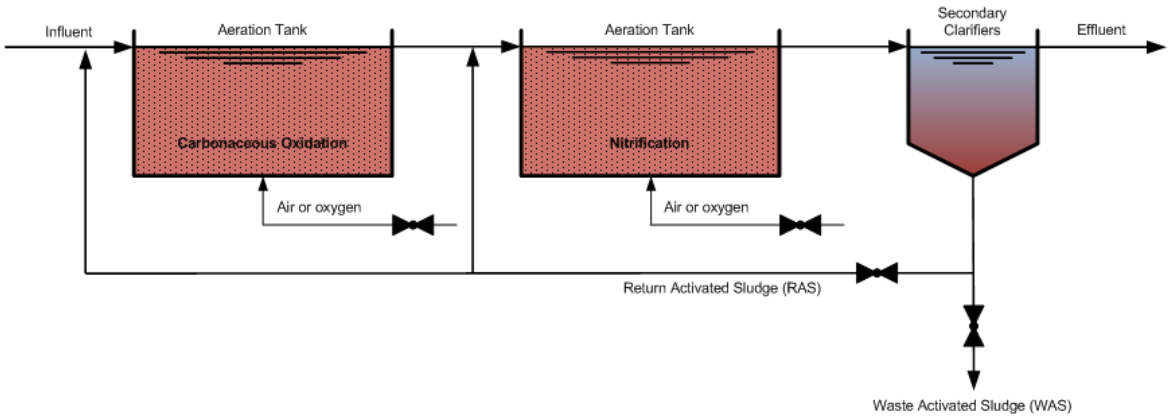


Figure 2-6: AS two-stage nitrification design (Adapted from Fuller 2017)

The single stage system was seen to produce less sludge than the two-stage design whose requirement of more reactors, clarifiers and aerators, made it more expensive to run than the single stage (Stover and Kincannon 1976). The SRT to achieve nitrification with single stage system was 10days while with two stages sludge retention time was 6 days thus aeration energy demand was less with two than one stage (Burton *et al.* 2014).

Also, with the two-stage system, toxic substances in wastewater which could be destructive to nitrifiers could be biodegraded (if biodegradable) in first tank and will have no effect on nitrification in second tank but in single stage system nitrifiers will be affected by toxic substances. For example, in two stage system, heavy metals may adsorb to sludge and settle away hence will not affect nitrifying organisms in first reactor. More so this reactor could act as a buffer to organic load impacts in the nitrification system in two-stage system as the effect of heterotrophic activity will be greatly minimised in first reactor. Conversely the rapid growth rates of heterotrophs in one stage system hampers nitrification (Stover and Kincannon 1976)

2.5 The effects of nitrification: Nitrite Toxicity

Nitrite is an intermediary compound of nitrification (section 2.2.1), but also occurs as a result of other processes like denitrification and dissimilatory or assimilatory nitrate reduction to ammonium processes in WWTS (Philips *et al.* 2002). In wastewater treatment systems its concentration is normally minimal and its presence transient (Pak *et al.* 1996). However, when steady state processes are disrupted or in fast moving systems, there is the availability of elevated concentrations of nitrite. In the latter, this has been attributed to nitrification but in slow moving conditions it has been attributed to denitrification (Kelso *et al.* 1997; Philips *et al.* 2002). During nitrification hampering or limiting nitrite oxidation results in the accumulation of nitrites. This usually occurs when ammonia oxidation rate is higher than nitrite oxidation rate even if the difference is just 3% (Smith *et al.* 1997a). This situation is typical of close culture systems with insufficient ammonia removal (Voslarova *et al.* 2008).

In nitrification, AOB which are normally said to have a higher yield, grow first as micro-colonies so that the presence of their product (nitrite) stimulates growth and establishment of NOB resulting in initial nitrite build-up in nitrifying bioreactors (Schramm *et al.* 2000). As nitrifying biofilms start forming, peaks of nitrite are observed as a result of slower growth of NOB to AOB (Phillips *et al.* 2002) at the beginning of the nitrification process. However, Randal and Buth (1984) stated that nitratation rate is eventually higher than nitritation so that in fully established systems this build-up is not observed. However, in WWT process designs whereby ammonium is intermittently added to the system, peaks of nitrite occur each time as AOB activity occurs. More so, due to the reduced growth rate of NOB nitrite accumulation occurs (Venterea and Rolston 2000) hence the two-step nature of nitrification itself contributes to its accumulation (Philips *et al.* 2002).

AOB and NOB have been observed to be associated with one another in treatment systems e.g *Nitrosomonas* closely associated with *Nitrobacter* in a continuously stirred tank (Schramm *et al.* 2000). This makes it possible that ammonia oxidising activities are linked up with nitrite oxidising activities both spatially and with respects to rate of activity (Philips *et al.* 2002). A disturbance of this spatial association of AOB and NOB, which hampers the mutual benefit that both nitrifying bacteria derive

from one another as mentioned in section 2.3 will distort balance of concentrations of nitrite thereby leading to accumulation of nitrite. Furthermore, in AS, aquaria and some freshwater systems, the more common NOB usually present is *Nitrospira* (Burell *et al.* 1999; Daims *et al.* 2000) so that researchers believe that absence of *Nitrospira* in a system will lead to accumulation of nitrite. Also, other research identifies nitrate at high concentration to be inhibitory to the activity of nitrite reductase enzyme thereby leading to accumulation of nitrite in the system (Kelso *et al.* 1997). Okabe *et al.* (1999) observe a spatial association of nitrifiers and denitrifies in WWS so that heterotrophs coexisted with nitrifiers benefiting from the organic products produced by AOB and the denitrifying heterotrophic ones use up nitrite produced. Therefore, any situation resulting in dissociation of this cohabing state will lead to accumulation of nitrite

Another possible reason for nitrite accumulation has been observed to be the presence of fermentative bacteria which may carry out dissimilative nitrate reduction (DNRA) to ammonium (Philips *et al.* 2002). In wastewater systems obligate anaerobes like *Clostidium* spp, *Desulfovibrio* spp and facultative anaerobes like *E. coli, Citrobacter* spp, *Salmonella typhimurium*, *Klebsiella* spp, *Enterobacter aerogenes*, as well as aerobes like some *Pseudomonas* spp. have been observed to carry out DNRA (Tiedje 1988, Cole 1996) and any hindrance to their activity results to accumulation of nitrite.

The disruption of processes like the conversion of nitrate to ammonium by the nitrate reductase gene (Bron and Zehnder 1990), increase biomass concentration as a result of nitrate assimilation (Philips *et al* 2002; Li and Irvin 2007) and presence of denitrification (Cole 1996) are sources of nitrite accumulation in WWTS.

2.5.1 Effects of nitrite presence

The presence of nitrite in biological processes has caused bacteriostatic effects as it associates with metals in enzymes thereby hampering enzyme-controlled reactions (Wild *et al.* 1995). It is said to be toxic in aquatic systems as its presence inhibits aquatic life (Philips *et al.* 2002). This toxicity has resulted in limitations to nitrite concentration in drinking and surface water to 0.05mg/L (EU C.D. 1998) and 0.03.mg/L NO_2^- respectively (Phillips *et al.* 2002). Lotti *et al*. (2012) state that its action could be reversible inhibition, whereby catalytic activity of the organism

involved is affected temporarily or toxicity, whereby an irreversible reduction in microbial activity occurs. Other researchers believe that nitrite toxicity is caused by the formation of nitric oxide (NO) or nitrosyle specie through the action of ammonia monooxygenase on nitrite so that a nitrosyle-metal compound is formed by an iron or copper molecule close to an enzyme site (Wu *et al.* 2013). NO, is very reactive and its toxicity is due to the fact that it destroys metabolic enzymes and reacts with superoxide to form peroxynitrite which is very toxic (Bredt 1999).

In WWTS nitrite accumulation has been observed to inhibit ammonia oxidation, nitrite oxidation, denitrification, phosphate removal, methanogenesis and cell growth (Phillips *et al.* 2002). This inhibitory effect is as a result of its ability to increase the proton permeability of cell wall so that ATP synthesis, hydrolysis and reactions catalysed by ATPase decrease.

This accumulation of nitrite and nitrate products of nitrification is of importance in WWTP as it stimulates the growth of filamentous organisms in nutrient removal systems where low F/m ratio exist (Musvoto *et al.* 1999). Here, floc forming facultative organism which denitrify nitrate to nitrogen gas are prevented from uptaking O_2 by the presence of NO which accumulates intracellularly and interacts with cytochrome oxidase, forming a Fe-NO complex which is not able to transfer electrons to oxygen hence the inhibition of aerobic respiration (Casey *et al.* 1999). Floc formers are not able to utilise their substrate and so move to denitrification of nitrite, which is a limiting condition. Filamentous organisms now take advantage of this floc limiting conditions and proliferate (Casey *et al.* 1999) so that effluent is not clear as floc formation and settling of sludge is hampered. Apparently, occurrence of bulking is dependent on sludge constituents but adding 0-100 mg/l of nitrite could stimulate bulking (Philips and Verstraete 2000).

Nitrite accumulation is however useful in other nitrogen removal processes in WWT whereby nitrification is stopped at nitrite and either nitrite oxidisers are washed out as in the single high ammonial removal over nitrite (SHARON) process or oxygen supply is controlled as in the anaerobic ammonium oxidation (ANAMMOX) and oxygen limited autotrophic nitrification denitrification (OLAND) (Phillips and Verstate 2000).

2.5.2 Factors affecting nitrite accumulation

Temperature, pH and nutrients which influence growth and activity of microorganisms' influence nitrite accumulation (Phillips *et al.* 2002).

2.5.2.1 pH

Information regarding the influence of pH on nitrite accumulation is inconsistent as different authors came to different conclusions. Observations of nitrite accumulation above 250-900 mg/L were made in sequence batch reactors when pH of mixed liquor increased from 7.5-9 in nitrifying system (Surmacz-Gorska *et al.* 1997; Glass and Silverstein 1998). Conversely, Tonkovic (1998) who compared sewage treatment plant data to lab test results said that nitrite accumulation was independent of pH but was a result of low dissolve oxygen while Furumai *et al.* (1996) observed nitrite accumulation at pH <7.4.

2.5.2.2 Free ammonia (FA/NH_3) and ammonium (NH_4^+)

Both NH_4^+ and NH_3 have been observed to cause accumulation of nitrite (Smith *et al.* 1997b) but the effect of FA was considered more pronounced (Phillips *et al.* 2002) as FA inhibits the nitrite oxidoreductase enzyme activity. FA inhibits NOB under the influence of initial NH_4^+ concentration, pH and temperature (Balmelle *et al.* 1992) but the amount of inhibition observed was less at temperature10 to 20°C. However, at ammonium concentrations ≤ 40 mg/L AOB and NOB become adapted to FA and nitrite build up is transient (Phillips *et al.* 2002).

2.5.2.3 Hydroxylamine

It was observed to be severely toxic to *Nitrobacter* (Hu 1990) resulting in irreversible nitrite build up in activated sludge batch reactors. Philips *et al.* 2002 stated that a correlation existed between accumulation of nitrite and concentrations of hydroxylamine and therefore attributed the presence of free hydroxylamine to be responsible for nitrite accumulation in batch reactors much more than FA and low dissolved oxygen concentrations.

2.5.2.4 Temperature

Tonkovic (1998) observed the accumulation of nitrite in activated sludge reactors during summer months (June-September) signifying the influence of temperature on nitrite presence. Also, at temperatures between 10 to 20°C the specific growth rate of NOB *Nitrobacter* was higher than for AOB *Nitrosomonas* but at 25°C the specific

growth rates were observed at about same range (Philips *et al.* 2002). However above 15°C another research observed higher growth rate of AOB to NOB (Mulder and Van Kempen 1997). Temperature change affects the relationship between the growth rates of AOB and NOB because the optimum temperature for nitritation is higher than for nitratation (Wortman and Wheaton 1991) so at high temperature nitrite oxidation is inhibited. This was further explained by Fdz-Polanco *et al.* (1994) that rising temperature stimulated the presence of NH_3 which inhibits NOB more than AOB.

Conversely, temperatures <14°C were found to cause nitrite accumulation in activated sludge due to reduced NOB activity below those temperatures (Randall and Buth 1984) but this was refuted by Mauret *et al.* (1996) as they did not observe any effect of temperature on nitrite accumulation. Instead they observed that as NOB activity reduced at that low temperature AOB activity reduced also proportionately.

2.5.2.5 Dissolved oxygen (DO)

This is very important for nitrification as both AOB and NOB are aerobic organisms. The DO saturation constant is dependent on density of biomass, floc sizes, mixing intensity as well as rate of diffusion of oxygen through floc (Munch *et al.* 1996). Leu *et al.* (1998) noted that oxygen saturation constants were 0.25 to 0.5 mg/L for AOB and 0.34 to 2.5 mg/L for NOB so that under conditions of low oxygen concentration AOB activity would continue while NOB activity would be reduced leading to accumulation of nitrite. At transient states of low oxygen concentration AOB activity has been observed to be dominant over NOB activity due to higher AOB affinity for oxygen in a mixed culture system as was observed for *N. europaea* and *N. winogradsky* (Laanbroek and Gerards 1993). In another experiment Hunik *et al.* (1994) observed *Nitrosomonas europea's* oxygen saturation constant to be 0.12 mg/L while that of *Nitrobacter agilis* was 0.54 out of limits stated above and this was explained by the fact that oxygen saturation was different at water phase, sludge floc or biofilm (Phillips *et al.* 2002) so that the effect of nitrite presence in each media would also be different.

To prevent nitrite accumulation in suspended growth systems bulk oxygen to bulk ammonia ratio should be monitored and should be at least 5 (Ceçen and Gönenç

1995). This has led some researchers (Joo *et al.* 2000) to conclude that indirectly it is oxygen concentration that causes nitrite accumulation rather than ammonia.

2.5.2.6 Organic matter

Organic matter increases the abundance of heterotrophs which outcompete nitrifiers (Zhang *et al.* 1995) for oxygen. The addition of organic matter therefore limits nitrifier activity and low oxygen concentration affects NOB much more than AOB such that though activity of both is limited, NOB are more affected (Okabe *et al.* 1999) hence accumulation of nitrite.

2.5.2.7 Operator parameters and treatment plant

Low solid retention time resulted in high nitrite in effluent as time for nitrite oxidation was limited but nitrite accumulation was also observed at when substrate concentration was high (Hanaki *et al.* 1990). However, competition for oxygen at start of nitrification process causes this accumulation to be retarded in nitrifying systems (Zhang *et al.* 1995) due to the predominance of heterotrophic activity. Rols *et al.* (1994) showed that inhibition of NOB activity by FA was dependent on the characteristics of water, flow regime (mixed or plug flow) as well as the WWTP operating mode (batch or continuous).

This section reveals that nitrite presence is inevitable in nitrifying system and affects their functioning due to its effect on microbial activity but could be monitored and controled

Chapter 3. Literature Review: Pathogen removal in Municipal Wastewater Treatment

3.1 Introduction

As wastewater treatment proceeds pathogens are slowly reduced (Bentecourt and Rose 2006). Pathogen removal routes in WWTS have been observed as physical, chemical and biological depending on type of treatment process (Bawiec *et al.* 2016), type of pathogen and even strain of pathogen (Rhodes and Kator 1988). Physical methods such as sedimentation, temperature, ultraviolet radiation and filtration, chemical methods including coagulation and chlorination and biological methods like natural die off, competition and predation have been observed in biological treatment systems (Sundaravadiviel and Vigneswaran 2001; Weber and Legge 2008). In order to understand these removal processes, the different type of pathogens, their survival and removal processes will be considered in the following sections.

Wastewater faecal pathogens are of gastrointestinal source, with their density dependent on contributions from infected populace to flow (Ottosons 2005). Table 3.1 gives examples of faecal pathogens that could be transmitted through water and improper sanitation. Their change of habitat from primary to secondary stage in the wastewater treatment process brings them in contact with different physico-chemical and biological conditions, which affects their growth and survival (Gordon *et al.* 2002) hence their density. Their reduction from wastewater is important to prevent possible transmission of diseases to individuals by inhalation or egestion when treated wastewater is reused for agricultural, selfish and bathing water purposes (Fu *et al.* 2010). Pathogen reduction is assessed by monitoring the concentrations of specific organism known as indicator organism (Anderson *et al.* 2005).

Table 3Examples of waterborne pathogens (Adapted from Ottoson 2005)

Pathogen Group	**Pathogen**
Helminths	*Ascaris lumbricoides*
Parasitic potozoa	*Giardia intestinalis*
	Entamoeba histolitica

	Cryptosporidium parvum or Cryptosporidum hominis
Virus	Rotavirus
	poliovirus
	Hepatitis E
	Hepatitis A
	Echovirus
	Enterovirus type 68-71
	Coxackievirus
	Calicivirus
	Astrovirus
	Enteric adenovirus 40 and 41
Bacteria	*Vibrio cholerae*
	Shigella spp.
	Salmonella typhi/ paratyphi and *Salmonella* spp.
	Yershinia spp.
	Plesiomonas shigelloides
	Escherichia coli (EIEC, EPEC, ETEC, and EHEC
	Aeromonas spp.
	Clamphylobacter jejuni/coli

3.2 Indicator organisms

Indicator organisms are markers of faecal contamination (Hai *et al.* 2014) in water and wastewater effluents. They are used to assess the possibility of pathogen presence in water samples because of time constrain and cost associate to the identification of individual pathogens (Ashbolt *et al.* 2001). Water and Wastewater management utilities assume that there is a relationship between indicator organisms and pathogens in a bid to prevent the persistence of pathogens to avoid the spread of waterborne infections (Harwood *et al.* 2005). Commonly used marker in assessing the bacteriological quality of treated water is the concentration of

coliforms (Mara 2003). These are considered as either total or faecal coliforms; such that total coliform (TC) are derived from faeces, soil and ubiquitous origins whereas faecal coliforms (FC) are derived from human and other warm-blooded animals and their number provides a check for faecal pathogens (Mara 2003). Characteristics of indicators include, easily and rapidly detectable, having the ability to survive longer than most viable pathogens, being resistant to environmental factors and disinfection as the actual pathogen and being a member of the intestinal flora (WHO 1993; Mara and Horan 2003; Bitton 2005). The presence of faecal coliforms has been shown to strongly correlate with the presence of faecal matter (EPA 2004) however total coliforms inhabit ambient water and could be affected by environmental factors that affect pathogens.

Commonly used indicator organisms include, *E. coli*, *Clostridium perfringens,* faecal streptococci*,* intestinal enterococci*, Bifidobacterial* and *Bacteriodes* (Ashbolt *et al*. 2001). Thermotolerant coliforms which are capable of being cultured at high temperatures like *E. coli* (44.5°C) are the main indicators in the water industry. *E. coli*, has been observed to be directly connected to sewage pollution and has also been identified as of faecal origin making it a reliable indicator (Ottoson 2005).

Enterococci are alternatives to coliform bacteria being more resistant to environmental stress and are useful in wastewater assessments as an indicator of enteric viruses especially in seawater and sludge (Bitton 2005). They've been used as indicators of contamination in bathing waters where their presence correlated with health risk associated. However, their use as indicator is limited due to their greater sensitivity to detergents than pathogens (Ottoson 2005).

However, there are limitations in the use of indicators. Protozoan and viral pathogens are less sensitive to inactivation and treatments processes than thermotolerant coliforms, are of shorter survival than bacterial pathogens and are not all faecal source. Also, the correlation of indicator with pathogens pathogen presence has been low (Girones *et al.* 2010, Ottoson 2005). Moreso, some indicators are pathogenic e.g. *E. coli* O157:H7 responsible for waterborne diseases of recreational waters (Ackman *et al.* 1997). Previous research (Ashbolt *et al.* 2001) state that no correlation exists between indicator organism and enteric pathogens so that for water quality purposes indicators were separated into three groups: Process

indicators which indicate the efficiency of a process e.g. total coliform indicating chlorine disinfection, faecal indicators which highlight faecal contamination by inferring that pathogens might be present e.g. *E.coli* indicating that pathogens are present and model or index indicators referring to specie or group of organisms whose presence is indicative of the presence of pathogenic organism. e.g. *E. coli* presence indicating the presence of *Salmonella*.

Limiting to the use of coliforms as indicators is the fact that they do not relate with protozoan and viral pathogens hence other indicators are used for these groups of microorganisms (Harwood *et al.* 2005). Process indicators for pathogenic enteric viruses are bacteriophages whose morphology and characteristic of survival are identical to those of enteric viruses (Turner *et al.* 1995). Their presence has been known to indicate the presence of faecal contaminated wastewater (Havelaar *et al.* 1986). Example of indicators of viruses are phages of somatic coliphages, *bacteriodes flagelis* and F-specific RNA bacteriophage also indicating faecal contamination (Ottoson 2005). Conversely, their use as indicators is debated as their mode of transportation and removal in wastewater treatment systems is observed to be dissimilar to those of enteric viruses (Xagoraraki *et al.* 2014).

3.3 Different Wastewater pathogens

3.3.1 Bacterial pathogens

Most common waterborne pathogens are bacteria pathogens whose ability to rapidly multiply under suitable conditions makes them easily detectable in wastewater samples (Okoh *et al.* 2007). Bacteria pathogens are 0.6-1.2 µm (Zhang and Farahbakhsh 2007) and organism such as *Staphylococcus aureus, Salmonella* spp*, E. coli, Clostridium perferingens and Listeria monocytogenes* have been shown to cause outbreaks of disease. Enteropathogenic bacteria are responsible for gastrointestinal infections like diarrhoea (of cholera by *Vibrio cholera)*, dysentery *(Shigella & Salmonella* spp), typhoid (*Salmonella* spp), human enteritis, legionelliosis, meliodosis, cancer and stomach ulcers are common diseases associated with wastewater borne pathogens (Le Chevallier and Au 2004; Liang *et al.* 2006). Bacteria pathogens could be opportunistic causing infections at optimal condition particularly in vulnerable individuals (babies & elderly) like *Pseudomonas* and *Streptococcus spp. Campylobacter* has been associated with sewage sludge

and surface waters (Sahlstrom *et al*. 2004). Episodes of water contamination have also been linked to *Shigella & Yeshina* spp. (Sharma *et al.* 2004). Bacteria pathogen resistance to adverse condition, is attributed to intrinsic factors like ability to associate with particulate organic matter, cell wall with waxy materials, ability to aggregate, low hydrophobicity and small size (Nwachukwu and Gerba 2004).

The identification of bacteria in wastewater effluents of municipal wastewater treatment systems is of health importance. Significant is the occurrence of members of *Enterobacteriaceae* and *Salmonella* spps. in wastewater effluents of surface waters due to the potential of causing diseases (Budzinska *et al.* 2014). Their presence is subject to the quantities of pathogens in wastewater stream. This is dependent on the occurrence of pathogens in domestic discharges of inhabitants connected to the sewerage system and their survival through the treatment process (Langelange 1982). However, though considerable reduction in pathogens is said to occur through treatment processes (Bentecourt and Rose 2006), dehydration due to treatment process causes the concentration of pathogens to be high (Budzinska *et al.* 2014). Also, relatively high quantities of *E. coli*, *Salmonella*, Coliforms and *Vibrio* spp. were identified in association with floc than in water (Danovaro *et al.* 2009; Droppo *et al*. 2009) in aquatic system therefore diseases are caused by persistent ones that pass-through treatment system.

The wastewater treatment technology as well as organic load are said to affect the chemical structure of the wastewater and are indirectly responsible for the quantity and diversity of pathogens in the system (Gobena *et al.* 2018) hence faecal coliforms. In the treatment of municipal wastewater important organisms include, *Pseudomnas aeruginosa, Pseudomonas fluorescens, Proteus vulgaris*, *Bacillus cereus, Enterobacter cloacae, Bacillus subtillis* all responsible for putrefaction, faecal indicators *Clostridia*, *E. coli,* and *Enterococcus* as well as human pathogens *Salmonella* spp.*, Shigella* spp.*, vibrio* spp.*, mycobacterium* spp., *Brucella* spp.*,* and *Legionella* spp*.* (Mosteo *et al.* 2013).

Salmonella and members of *Enterobacteriaceae* family were identified in effluents of sewage treatment despite the observed efficacy of the system (Budzinska *et al.* 2014). *Salmonella* spp., *Esherichia* spp., *Shigella* spp., *Vibrio* spp., *Camphylobacter* spp., *Clostridium perferingens*, *Faecal streptococci, Enterococci* were observed in

effluent municipal wastewater treatment effluents after AS treatment (Naidoo and Olaniran 2013).

3.3.2 Protozoan pathogens

Pathogenic protozoa in wastewater are more prevalent than from any other environment and include *Entameoba*, *Gardia, Cryptosporidium Cyclospora*, *Toxoplasma* and *Microsporidia* species (Girones *et al.* 2010) with *Cryptosporidium* and *Giadia* spp. reported to be of highest resistance than all others (Cassio *et al.* 2003) to disinfection. 95% of cases reported of human cryptosporidiosis have been linked to *Cryptosporidium parvum* and *Cryptosporidium hominies* (Xiao and Ryan 2008). *Gardia* spp. is associated with diarrhoea (Reynold *et al.* 2008). These are said to survive outside their host as cyst and oocyst of between 3-14µm in diameter (Ottoson *et al.* 2006) hence their ability to persist in adverse conditions.

3.3.3 Virial pathogens

These are the most hazardous waterborne pathogens, as they are more resistant, more infectious even in small numbers and more difficult to detect than other environmental pathogens (Okoh *et al.* 2007). Enteric viruses are the most commonly found viruses in municipal wastewater treatment systems (Cai and Zhang 2013) and nose, throat and blood fuilds are also sources of wastewater viruses (Mara 2003). These get into wastewater by faecal contamination and human household fluids from infected individuals (Leclerc *et al.* 2000). They are said to be more resistant to treatment, more infectious, more difficult to detect and could cause infection at low concentrations (Gomez *et al.* 2006). A common threat to WWTP workers is the hepatitis A virus (Cai and Zhang 2013). Others common viruses are strains of Enteroviruses, Echoviruses and Coxsackievirus (Yasunori *et al.*, 2002) and these pose a risk particularly to children and elderly. Viruses transmitted by wastewater are said to be stable because they've already resisted the digestive track condition and lack a lipid coating which makes other viruses susceptible to environmental agents (Girones *et al.* 2010). However, resistance of viruses to water treatment is attributed to possession of a double stranded DNA and their small size (Nwachukwu and Gerba 2004).

3.4 Processes for pathogen removal

Municipal wastewater is rich in nutrients providing an environment whereby floc and suspended particles by electrostatic attraction concentrate dissolve nutrients around them. This makes them rich and viable for attachment of microorganisms hence pathogens (Malham *et al.* 2014). Pathogen growth is dependent on nutrient availability and extensive growth of *E. coli* and enteric bacteria has been observed in organic rich aquatic environments (Wanjuyi and Harwood 2013). Parallel studies show that aquatic systems with particulate matter support the survival of pathogens much more than clearer aquatic systems due to the interaction of suspended matter and pathogens which is even enhanced by presence of nutrients (Malham *et al.* 2013). A summary of pathogen removal processes relevant to municipal WWT is given in table 3.2 and in the next sub-sections details of pathogen removal processes and their relevance in WWT is discussed.

3.4.1 Flocculation

Floc are sediment in the water column formed as a result of Brownian motion, differential settling abilities of particles, fluid shear as well as the interaction of organic and inorganic suspended particles with the help of extra polymeric substances (Droppo 2001; Wotton 2004). Their formation is enhanced by low to medium mixing as well as high concentrations of suspended matter (Law *et al.* 2013). These are multifaceted and transient structures which are very important components of suspended matter biogeochemical cycles and reflect the components of the aquatic environment in which they are formed (Malham *et al.* 2014). Low to medium mixing as well as the presence of a high concentration of suspended matter enhances flocculation (Law *et al.* 2013). The particulate organic material in floc are converted to dissolved organic material by extensive hydrolysis so that dissolved organic matter remains in water column available for mineralisation while particulates aggregate and sediment. Floc possess a highly negative surface charge density which makes it possible for pathogens to get attached to them and are attracted to aquatic bacteria because of their carbon content (Azam and Long 2001). Pathogen attachment to floc is promoted by the presence of extracellular polymeric substances and in previous research, relatively high quantities of *E. coli*, *Salmonella*, Coliforms and *Vibrio* spp. were identified in association with floc than in water (Droppo *et al.* 2009; Danovaro *et al.* 2009). Floc presence increases the downward flow of

sediments hence the migration of pathogens associated to them. Recent research identifies floc as major reservoir for persistent human pathogens (Lyons *et al.* 2010) in aquatic system implying that they are relevant in pathogen reduction processes in treatment systems. The rate of adsorption of bacterial unto sludge floc is directly proportional to the rate of sedimentation as has been observed in coliform removal (Mara 2003).

The significance of flocculation in biological treatment is the formation of settleable particle aggregates whose sedimentation and ultimate separation from water gets rid of pollutants (Malham *et al.* 2014). These processes are influenced by the mode of bonding of floc, particle compositions, hydrodynamics, microorganism's isoelectric points and the biological environment of the aquatic system (Nwachcukwu and Gerba 2004; Fettwise *et al.* 2006; Law *et al.* 2013).

Due to the adsorption and association of pathogens to floc, surface densities are higher in floc than water column and this association provides a means of survival and transportation of pathogens (Danovaro *et al.* 2009; Droppo *et al* 2009). *E.coli*, salmonella, and coliforms which are associated to floc have been found to be seven fold more enriched with organic matter and survival ability than others without that interaction hence of significant health risk (Malham *et al.* 2014).This bacteria-floc interaction is a transient one as it is affected by breaking and rebuilding processes that are influenced by porosity, particle size, shape, density, particle size and environmental factors (Droppo 2001; Furukuwa *et al* 2014) all of which affect the ability of floc to settle or be resuspended hence moving associated pathogens around in the system. However, pathogenic viruses which have varying hydrophobic and isoelectric nature only bind to floc when their isoelectric point is close to pH hence their persistence in treatment environment (Nwachukwu and Gerba 2004).

3.4.2 Sedimentation

Sedimentation of suspended particles in wastewater treatment takes place by gravitational solid movement and removes settleable solids as well as other wastewater constituents which adhere on them such as nutrients and pathogenic organisms. After adsorption, survival of microorganisms depends on organic content, nutrient availability, predation, competition from other microorganism and environmental factors like pH and temperature (Malham *et al.* 2014). Their survival

therein is affected by structure of sediments. In previous research (Garzio-Hadzick *et al.* 2010) faecal bacteria associated with finer particles survived better than those with rough particle and less organic matter. These factors are important in primary and secondary sedimentation stages of treatment where available bacteria gets associated to sediment.

However, growth and survival of pathogens as a result of sedimentation is dependent on nutrient availability around the sediment to which the bacteria are attracted. This was seen in organic rich wastewater where it was observed that *E. coli* and other enteric bacteria showed increased growth (Anderson *et al.* 2005) at sediment site. Conversely in microcosm *insitu* studies on the interaction between nutrients and pathogens using artificially nutrient rich water, a net faecal bacteria decay was observed in the sediment. Researchers believed change could be due to protozoan predation which mask growth of pathogens, to the fact that only identified pathogens were persistent ones which avoided predations and to the fact that possibly the pathogens evolved into a viable but non culturable (VBNC) state which prevented them for being identified (Craig 2004). VBNC state has been observed in enteric organisms like *E. coli* and *Salmonella* spp (Oliver 2005). As when not in their normal gut environment, pathogen cell reacts by getting into a survival viable but non culturable state - VBNC in which they cannot be cultured on growth media but still have metabolic functions (Oliver 2005). However, these cells are able to return to normal state when conditions are favourable. Researchers have mixed opinions about these resuscitated cells due to difficulty in asserting the difference between resuscitated cell and cell obtained by regrowing cultivable cells obtained from a VBNC group of cells (Oliver *et al.* 1995). The implication of the formation of this state is that enumeration counts on microbial culture media using samples from these types of waters will not give a true representation of viable cells. This state could be induced by non ionic surfactants present in household detergents which could have been passed into water system. However Gram-positive organism are said to be more susceptible than Gram-negative ones (Robben *et al.* 2018)

Due to the association of organic and inorganic particles with sediment they've been observed to be reservoirs of pathogenic protozoa cyst e.g *Crptosporadium* spp. and *Giadia duodonalis* oocyst (Medema *et al.* 1998) hence they could be separated from surrounding water. However, these cysts are not metabolically affected by the

presence of nutrients. Rather, the charge and hydrophobicity of their surface is influenced by the ionic state and organic composition of wastewater thereby influencing their ability to associate with other particles hence their sedimentation or transportation within the system (Dumetre *et al.* 2012).

Viruses, on the other hand, are non-living entities and therefore not metabolically affected by nutrients. However, as nutrient presence enhances biofilm formation, flocculation and extracellular polymeric substance (EPS) formation, which are relevant to the attachment of pathogens to sediments, it indirectly therefore supports the survival of viral pathogens which absorb to them (Shapiro *et al.* 2013). This attachment protects them from salinity, temperature and even UV radiation (Malham 2014).

Sedimentation accounted for reduction of faecal coliforms and pathogens at secondary treatment as biomass is separated from effluent water in biological unit. This separation step separates pathogens attached to floc in sludge from effluent wastewater and because of no mixing greater adsorption to sludge floc increases as sedimentation occurs (WERF 2004). Secondary sedimentation in a biological treatment system will increase mixed liquor suspended solid content of the system hence the adsorption rate which should in turn increase removal rate of pathogens. Increase in mean cell residence time would also result in increased sludge formation so that more pathogen will likewise be removed (Nwachukwu and Gerba 2004). Faecal coliform and salmonellae removal of 90-99%, 99% of *Campylobacter,* as well as no survival of *V.cholorae* in activated sludge systems that are in operation are reports of pathogen removals in suspended growth systems mainly by sedimentation as a result of the formation of settleable bacteria floc, adsorption to surfaces and predation processes which are all dependent on the hydraulic retention time (Curtis 2003).

3.4.3 Ultraviolet radiation

Sunlight which is the main source of UV radiation has been identified as the most bactericidal agent in natural waters (Fujioka and Narikawa 1982). Sunlight intensity results in increase in temperature which itself is said to be directly related to predation as predation was found to increase exponentially when temperature

changed from 12-22ºC (Sherr *et al*.1988) in mixed assembly of flagellates and ciliates of marine protozoa.

The thermal property of sunlight has been effective in inactivating coliform bacteria at temperatures above 45°C (Berney *et al.* 2006). UV-A (400-315 nm) and UV-B (313-280 nm) waves reaching the earth and combining with temperatures between 45-60°C are capable of inactivating pathogens (Mtapuri-Zinyowera *et al.* 2009). This is due to an optical mechanism from the sun's UV radiation which breaks up molecular bonds in the microorganism's deoxyribonucleic (DNA) or ribonucleic acid (RNA) so that the cells are incapable of reproduction or infecting the host (Kendriks 2013). UV A also reacts with dissolved oxygen in water to form oxygen free radicals and hydrogen peroxide which are also said to interfere with cell structures and destroy pathogens (Meierhofer and Wegelin 2002).

UV disinfection could be achieved by exposure to sunlight, exposure to lamps with mercury and also solar pools. UV radiation has been effective in treating secondary and tertiary wastewater effluents (Lee *et al.* 2015) of bacteria, viruses and high protozoa oocyst (Taghipour 2004) removal. Adenovirus are the most resistant to UV radiation as they have a double stranded DNA which allows for the use of host cell enzymes during replication to repair damages caused by UV radiation (Kendricks *et al.* 2013). However, inactivation of pathogens in raw or highly organic wastewater is limited by the shielding effect of turbidity caused by the suspended particulates (Fujioka and Narikawa 1982). Also, it can only be applied in small quantities of water and the effect could be repaired or reverse if dosage is low (Hoyer 2003).

3.4.4 Coagulation

Wastewater is dose with high molecular weight and high charged chemical to induce flocculation (Jimenez *et al.* 2010). Normally it is done by chemically enhance primary treatment or advance primary treatment processes with four to six hours and half to one-hour retention times respectively implying that retention times are relatively shorter than other processes. Lime, ferric and alum coagulants have been used (Mara and Horan 2003). Salmonella (1 log), faecal coliforms (1 log), as well as 90-99% helminth ova reductions have been observed by coagulation (Jimenez *et al* 2010). 90-90% removal rates of protozoan cyst have been observed with coagulation

and this rate increases with increase in concentration of coagulant (Mara and Horan 2003) as well as 5 log removals of *Cryptosporidium* (Huck *et al.* 2000).

3.4.5 Filtration

Could be achieved biologically or physically and pathogens are removed as they pass through sand or granular matter pores, by retention in sieves, adsorption to surfaces, straining, interception and sedimentation processes (Jimenez *et al.* 2010). Slow and rapid sand filtration as well as the use of membrane are filtration systems employed in WWTS at both primary and secondary stages. Filtration is a size exclusion method and shows high pathogen removal especially for protozoa cyst (Mara and Horan 2003). Filtration is enhanced by adsorption which relies on the degree of interaction between the surface of microorganism and absorbent and this interaction is influence by their electrostatic and hydrophobic characteristics as in removal of viruses (Nwachukwu and Gerba 2004).

3.4.6 Chemical methods of pathogen reduction:

These chemicals are applied at tertiary treatment to treat secondary effluent before discharge into environment. Conventional chemical methods include chlorination, ozonation but UV radiation is sometimes considered chemical though it leaves no chemical by-products (Okoh *et al.* 2007). Microbial sensitivity to disinfection is influenced by the organism's ability to get attached to surfaces, low growth, encapsulation and aggregation (Le Chevallier and Au 2004).

Disinfection by chlorination is the most common chemical method of pathogen reduction in wastewater treatment worldwide (Huang and White 2006) and has been useful in treating secondary effluents (Wu *et al.* 2013). It could be applied to wastewater in gaseous, liquid and solid forms (Okoh *et al.* 2007). The bactericidal action of chlorine is achieved by the modification of the chemical structure of enzymes which are responsible for metabolic activity in bacteria resulting in impairment to growth, hence development as they are inactivated (Collivignarelli *et al.* 2017). However, in the presence of natural organic compounds carcinogenic compounds trihalomethane and acetoacetic acid are produced (Wu *et al.* 2013).

An alternative to chlorination is peracetic acid (PAA) and it releases active oxygen or hydroxyl radicals which disinfect by attacking cell wall, cell membrane, DNA and

enzymes (Lee and Von Gunten 2016). However, use of PAA increases BOD and COD of effluent (Collivignarelli *et al.* 2017)

Another chemical is Chlorine dioxide whose germicidal action is due to its high oxidative ability which enables it to inactivate pathogens either by disabling protein synthesis or by inactivating enzyme activity (Cho 2010). However, its disinfection action produces potentially toxic chlorite and chlorate by products.

Ozone another oxidising agent operates by destroying cell wall of pathogenic organisms. It is produced by electrolysis, electric shock producing radiochemical reactions and photolytic reactions (Collivignarelli *et al.* 2017) capable of destroying viruses and bacteria. Though an expensive method (Hijnen *et al.* 2004), the conversion of ozone to oxygen after disinfection creating no harmful product makes it acceptable (Okoh *et al.* 2007).

Table 4 Common Pathogen removal processes relevant to municipal wastewater treatment (Adapted from Jimenez *et al*. 2010)

Treatment Process	*Pathogen removal process*	*Design characteristics*	*Advantages*	*Disadvantages*
Waste Stabilization ponds	-Sunlight -Temperature -High pH>9.4 -High DO -Sedimentation -Adsorption -Predation	- Ponds in series -Retention times (5-20days)	-Good in both warm & cold climate -No electricity -Easy operation & maintenance	-Requires large areas of land -Loss of water increases salinity -vegetation growth could breed vectors
Septic tanks, Imhoff tanks, UASBs & High rate Anaerobic ponds	-Sedimentation	-retention time (6-12hrs)	-Little maintenance and operation	
Constructed wetlands	-filtration -adsorption -predation -Plant exudes	Retention times (4-6day) Beds in series	Little maintenance and operation	-mosquitoes -requires land
Primary sedimentation	Coagulation - flocculation Adsorption Filtration	Retention times short (0.5-6hrs)	-Useful for primary and secondary effluent -cheaper than AS	-small viruses and bacteria pass through - High sludge production -clogging

Activated sludge and different types	High DO (Bacteria) Sedimentation Adsorption Filtration	Medium retention times (4-8days)	Different type useful for primary and secondary effluent	High investment cost
Membrane reactors, Sand & rapid filtration	Filtration	Medium retention times	Efficient pathogen removal	-Expensive High maintenance
Chlorination and ozonation	Chemical inactivation	Short contact tines	No effect on protozoan cyst	-Low organic matter effluents -Chlorination by products are cacinogenic
UV radiation	Light, Temperature, DO	Different retention times	Efficient except for Adenoviruses	Only useful with low turbid waters

3.5 Biological Methods of Pathogen reduction

The main controls of bacteria population in wastewater treatment systems are protozoa grazing and bacteriophage attack but bacterial predation (Dolinsek *et al.* 2013), competition (Okoh *et al.* 2007) and natural die off have also been identified. Reduction of pathogens by vermifiltration (Sinha *et al.* 2008) has also been identified in natural systems whereby earthworms are predators of pathogens but also, their presence stimulates the growth of antibacterial microorganism which cause the death of indicator organisms (*E. coli*, faecal streptococci, *salmonella* and *shigella* (Arora *et al*. 2014). However, vermilfiltration is not relevant here as earthworm's presence will most likely occur at pre-treatment stages.

The wastewater system's community structure and coexisting biological diversity is affected by competition which interacts with other pathogen reducing mechanisms such as predation, natural die-off, and starvation (Okoh *et al.* 2001). Cloete and Muyiwa (1997) state that when organisms utilise the same substrates, competition for it determines the structure of the community and biological diversity. In wastewater systems available resources are rapidly utilised by fast growing *Pseudomonas* and coliforms spp. and thereafter bacteria including pathogenic ones get inactivated by endogenous respiration (Okoh *et al.* 2001).

3.5.1 Natural Die-off

Natural die-off was identified as a major route of pathogen removal in natural treatment systems like constructed wetland (Weber and Legge 2008) and is said to be correlated with hydraulic retention time (Kadlec and Knight 1996). Karim *et al.* (2004) found that the die-off rates of bacteria and coliphages were greater in the water column than with sediments but protozoa die-off rates were greater in sediments than in water column. Die-off in WWT is influenced by reduction in organic carbon, oxygen deficiency for aerobic organisms, competition and process design (Karim *et al.* 2004).

3.5.2 Predation

Protozoa are the main predators in WWTS and their presence has been shown to result in reduction of pathogens and production of good quality effluent hence their use as bio indicators of treatment effluent quality and process performance (Martin-Cereceda *et al.* 1999). Several studies have attested to the reduction of wastewater microorganisms by protozoa including reduction of slow growing bacteria, faecal coliforms, typhal, choleral, streptococcal and diphtherial bacteria (Sinclair and Alexander 1989; Espinosa-Garcia *et al.* 2014). This predation is depended on the size of bacteria and is characterised by selective feeding of prey which is dependent of size, growth conditions, motility of bacteria and type of treatment system (Harvey *et al.* 2002).

Protozoa predation was responsible for reduction in coliform numbers in the liquid part of septic tank effluent as well as the solid phase (Chabaud *et al.* 2006) and was also responsible for removal of faecal coliforms from sand filters treating fish wastewater (Bomo *et al.* 2004). As their predatory behaviour results in reduction of coliforms, they've been called disinfecting agents and in the assessment of sludge health and biodegradability some researchers consider them indicator organisms (Papadimitriou *et al.* 2010). Madoni's sludge biotic index associates the presence of small free-swimming ciliates to indication of moderate treatment in conventional AS systems (Madoni 1994). Also, ecotoxicological data have revealed that alongside bacteria evaluations, protozoa assessments are important in understanding pollutant dynamics of activated sludge systems (Pauli and Pauli 2014).

For effective predation, ciliate presence is critical as they consume bacteria as a source of nutrients (Lester and Beskett 1999) and evolve different forms well adapted to resist changing environment in treatment plant (figure 3.1). It was observed that physico-chemical parameters, nitrate, dissolved oxygen and electrical conductivity were very important in determining the distribution of ciliates in biological treatment processes in constructed wetlands and AS systems (Martin-Cereceda 1996). Protozoa are also relevant for nutrient cycling and carbon mineralisation in WWS (Madoni 2011). Their clarifying ability leads to selection of bacteria that can grow fast and inefficiently so that carbon utilization increases (Pogue and Gilbride 2007) but in conditions of intense predation slow growing bacteria are not spared (Sinclair and alexander 1989). This implies that selective feeding which is dependent on the physico-chemical structure of the bacteria surface, size of prey and predator could be responsible for the excessive loss in bacteria population.

Predation is by phagocytosis which involves filtration of wastewater that comes through by its ciliary sieves thereby retaining suspended particles and subsequently breaking down digestible matter and in so doing clearing the waters and creating an effluent which is less turbid (Macek *et al.* 1991). *Vorticella microstoma* and *tetrahyemena* frequently found in wastewater treatment plants have been shown to have high filtration rates (Hatzis 1993). This filter feeding by ciliates is non-selective such that both pathogenic and non-pathogenic bacteria concentrations have decreased e.g. diphtherial, cholera, typhoid and streptococcal bacteria in laboratory bench scale experiments (Pauli *et al.* 2001). However, the water clearing ability of protozoa is dependent on the size of prey as protozoa were observed to ingest more larger bacteria (Chrzanowski and Simek 1990). Seasonal variations were also observed e.g in bench scale AS systems running at temperatures $\geq$ 36°C decrease in ciliate concentration caused effluents to be turbid. Figure 3.2 shows the effect of protozoa on bench scale AS bacteria numbers (Pauli *et al.* 2001). It shows increase in growth of bacteria numbers in the absence of ciliates and decrease in growth and number of bacteria in the presence of different forms of ciliates.

Members of the Ciliophora group are the protozoa of greatest dominance (Papadimitriu *et al.* 2010) in the WWT and their presence is associated to biofilm formation and aerated zones (Pauli *et al.* 2001) as they are mostly aerobic organism. The presence and type of protozoa is affected by the content of the wastewater:

more flagellates are found in high organic content wastewaters. Protozoa quantities increased with increase in organic loading and oxygen in aerated CW systems (Zapater Pereyra *et al.* 2015) and reduced solid accumulation due to less biofilm accumulation by predation activity. Apart from their effects on bacteria number, protozoa excrete particle aggregating excretory products on which suspended bacteria form floc which settles (Taylor and Berger 1980) but this means of reduction in bacteria number by protozoa is regarded as minimal as bacteria on their own are able to excrete extracellular polymeric substances which enables formation ofx settleable floc (Stehr *et al.* 1995).

Also, protozoa in WWTP consume particulate matter of the size of the bacteria they can consume as well as macromolecules and this enhances effluent clarity. They've been observed to take in substances directly through their cell plasma membrane and by pinocytosis, like in *Tetrahymena* where uptake of amino acids and glucose was observed (Pauli *et al.* 2001).

Contrary to the positives of predation on wastewater treatment, the predator-prey relationship between protozoa and bacteria has been shown to reduce the pollutant reducing properties of biological treatment systems supporting an old view of protozoa being harmful to WWTS. This is because after a time of predation the concentration of bacteria remaining in the system reduces significantly so that the processes of organic carbon and nutrient biodegradation reduces. This has been observed in a model system where protozoa *Tetrahymena pyriformis* was grown in a batch system containing *E. coli* (Watson *et al.* 1981) and natural system (Saunders *et al.* 1989). However, predation is sometimes countered by the ability of bacteria to evolve mechanisms to resist ingestion by protozoa e.g. when aggregates of bacteria forms were observed in wastewater treatment plants (Pauli *et al.* 2001) or the spontaneous organisation of bacteria into floc biofilms in the presence of protozoa (Pauli *et al.* 2001). Ironic though, as floc formation in WWS is said to be instigated by protozoa presence and occurs at different degree with different types of ciliates (Macek 1991). Other researchers observed that in the presence of certain ciliates e.g *Cyclidium spp* phenotypically different types of bacteria, resistant to predation appeared (Shikano *et al.* 1990). Also, Gurijala and Alexander (1990) identified the protozoa *Tetrahymena thermophila* not feeding on bacteria with hydrophobic surfaces as this enhanced their ability to aggregate and difficult to digested.

As protozoa have an influence on the amount of microorganism, they indirectly influence the microbial control processes in aquatic systems. The next section discusses the possible effects of biological activities on nitrification in biological systems.

3.6 Biological Control of nitrification: Predation

Nitrifying organisms in AS systems exist in clusters as floc and together generate soluble microbial products and these combined with death nitrifying bacteria cells are said to support the growth of heterotrophs (Rittmann 1994). Heterotrophs make up 50% of bacteria in nitrifying systems that obtain energy and carbon from ammonia and bicarbonate respectively (Kindiachi *et al.* 2004). The presence of some of these heterotrophs affects the stability of nitrification in both natural and engineered systems as the act as bacteria predators (Dolinsek *et al.* 2013). Evidence of this was seen when florescence *insitu* hybridization analysis of AS reveal that *Nitrospira* diversity and numbers, hence nitrification was affected by its association with *Micavibrio* bacteria. These bacteria predators whose presence is associated to presence of nitrifiers were also observed to prey on pathogens (Darshiff *et al.* 2010) as well hence their presence led to enhanced faecal coliform removal. The grazing action of protozoa (Morenzo *et al.* 2010) and attack by bacteriophages (Choi *et al.* 2010) on nitrifiers are other biological activities that regulate nitrification in aquatic systems.

However, protozoa have been identified as being the main organisms responsible for reduction of bacteria (Malham *et al.* 2013) by their predatory activity which is indiscriminate of specie (Pauli *et al.* 2001). Predation is facilitated by its phagocytic mode of nutrition in which suspended particle and bacteria cell between the sizes of 0.3-5µm are filtered from surrounding water by cilia, concentrated and digested (Fenchel 1980). This protozoan activity which clarifies water, is more present in AS systems and more so, in AS systems possessing submerge fixed-bed filters as additional surfaces for slime growth as these supports the growth of sessile ciliates (Hu *et al* 1993). In these modified AS systems protozoan biomass has been observed to be 68% (dry weight) of total biomass as compared to 10% in normal activated sludge systems implying protozoan activity to be higher hence a clearer effluent produced (Hu *et al.* 1993).

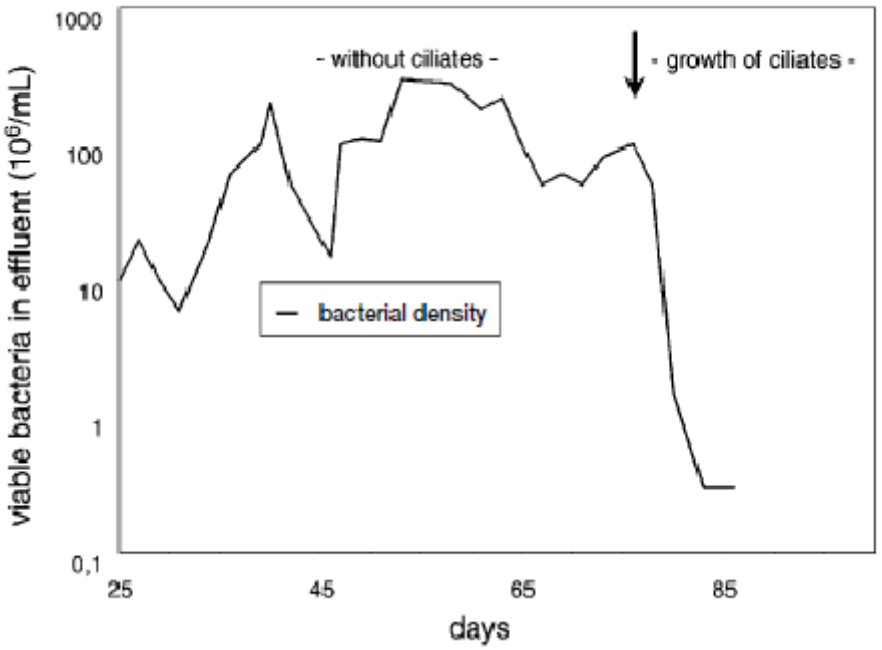


Figure 3-1: Effects of the presence of protozoa on bacteria content of bench scale AS effluent (Pauli *et al.* 2001)

Research by Petropoulos and Gilbride (2005) show that the presence of protozoa in a system increases the nitrification rates. Protozoa grazing reduces the population of fast-growing heterotrophs hence competition for oxygen and even ammonia. They also suggested that the assimilation of ammonia by heterotrophs occurred preferentially to nitrification in AS systems so that ammonia quantities available were reduced. Overgrowth of heterotrophs would therefore create nutrient limiting conditions which would reduce cell activity thereby reducing the efficiency of the AS systems. Ironically protozoa which consume live cells have been found to excrete the largest amounts of inorganic nitrogen in aquatic systems (Sherr *et al.* 1988) so that the presence of protozoa has been observed to lead to increase in the concentration of ammonia in aquatic systems due to their metabolic activity (Petropoulos and Gilbride 2005). Conversely, other research (Sherr *et al.* 1988; Sinclair and Alexandar 1989) suggest that protozoa had the effect of eliminating slow growing nitrifying bacteria in non-flocculated WWTS so that nitrification is reduced but in AS system these bacteria are associated with floc (Wagner *et al.* 1995).

Testae ameobae are examples of protozoa found in nitrifying activated sludge systems (Madoni 2011) and their presence is said to have an influence on the morphology, taxonomy and physiology of bacteria communities in these systems (Hahn and Hofle 2001) hence nitrification. Some research suggests no impact of protozoa presence to nitrification (Lee and Oleszkiewicz 2003; Sherr *et al.* 1988) but in model systems, the presence of protozoa stimulated nitrification activity (Verhagan *et al.* 1995). In fact, protozoa predation was the main cause of failure for the bioaugmented nitrifying system (Bouchez *et al.* 2000) as growth of protozoa was linked to reduction of added nitrifying bacteria in the reactor.

A suppression of predator activity should increase bacteria availability and activity hence nitrification as estimated in a model linking heterotrophic, autotrophic and predation activity in AS system (Moussa *et al.* 2005). A negative correlation between crawling and sessile ciliates and suspended matter resulting in high BOD of effluent when protozoa was absent resulting in low effluent qualities was observed with full scale AS plants treating municipal wastewater and even brewery waste (Salvado 1995; Fernandez-Leborans and Moro 1991). Also, mechanical reduction of protozoa activity using ball mill to destroy protozoa in influent wastewater was observed to result in ten folds increase in *E. coli* in wastewater effluent concentration (Pauli *et al.* 2001).

On the other hand, in an experiment with bench scale plants treating municipal wastewater, ciliate activity showed no effect on nitrifiers in floc, were responsible for an increase in degradation of soluble organic matter, contributed to the presence of sludge and were responsible for an increase in biodegradation of organic pollutants like 2,4-dichlorphenol. Chudoba (1985) explain this as a result of the breakdown of bacteria metabolic products like acetic acid, butyric acid and ethanol hence preventing end-product inhibition activities in the system. This was also explained by ciliates releasing organic substances like amino acids and growth stimulating factors (Andersson *et al* 1985; Bloem; Bar- Gilissen 1989) capable of stimulating bacteria activity (Henkinet *et al.* 1990). Compared to other zooplankton, protozoa excrete the highest amount of inorganic phosphate and nitrogen (Sherr *et al.* 1988) but these additions of nutrient are said to be more relevant in industrial and commercial wastewaters than in domestic waste waters (Pauli *et al.* 2001).

A generally succession of protozoan organisms from flagellates, naked amoeba to ciliates or from swimming, crawling unto more sessile forms in stabilization stage was observed in AS systems treating municipal wastewater. This succession was attributed to wastewater composition changes with time by the reduction of organic carbon concentration (Curds 1992; Madoni 1994; Salvado 1994). Figure 3.2 present protozoa concentration in the AS treating domestic sewage with time. Type and concentration of protozoa changes as treatment proceeds. Madoni (1994) noted that increase in sludge and biofilm was proportional to increase in ciliate biomass implying that ciliates and bacteria have a close relationship hence the influence in biological controlled reactions.

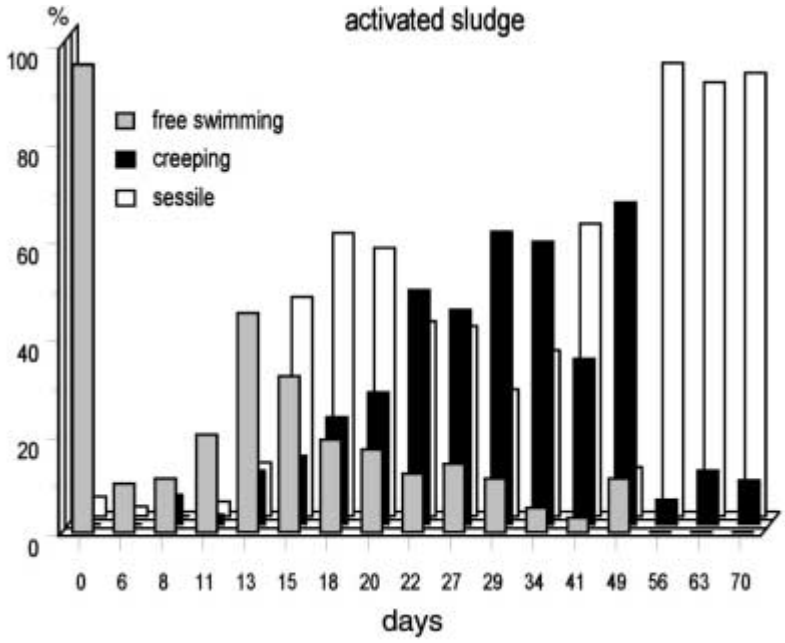


Figure 3-2: Change in protozoa type and concentration in a fullscale AS systems (Madoni 1994a)

The wastewater organic load has a influence on the ciliate population density, species diversity and more diversity were observed in wastewaters characterised by low organic carbon (Pauli *et al.* 2001). Ciliate activity was observed to be optimal between 10-25°C but their concentration decreased at temperature above 30°C and inactivity at temperatures above 40°C was observed. Growth and propagation of protozoa is favoured by pH between 6.5 and 8 which are normally present in AS

systems. High concentrations of protozoa are precedent of aerobic conditions and very few facultative or anaerobic species have been observed. (Pauli *et al.* 2001).

3.7 Summary of Literature Review

In assessing the factors that affect the biological processes of nitrification and pathogen reduction in municipal wastewater systems the review above has considered nitrification and its effects on microorganism in the system, pathogens, possible routes of reduction and the effects of protozoan predation on the bacteria numbers in the system.

Pathogens are of bacterial viral and protozoa forms and the process of nitrification is carried out by bacteria agents. This implies that factors that affect the growth and activities of bacterial organisms in the wastewater treatment systems will affect both processes as these organisms are interacting in same environment. The interaction of these organisms brings us to the hypotheses that the processes of nitrification and pathogen removal are not mutually exclusive.

Research shows that nitrifiers are slow growers and very sensitive to environmental change much more than heterotrophs. However, heterotrophs are capable of out competing nitrifiers for oxygen needed for biodegradation of organic matter so that in the municipal wastewater system organic carbon degradation take place before degradation of inorganic nitrogen. This means an average of 8-12 days of solid retention time of mixed liquor in the biological chamber with aeration is needed to achieve both processes. Nitrifiers are more sensitive to environmental factors like pH, temperature and dissolved oxygen so these physical characteristics must be monitored when aiming to nitrifying wastewater. However, during the ongoing nitrification process pathogens are in the system and as living entities would be affected by the effect of aeration which is important for both organic carbon degradation and nitrification. Many authors have writing about nitrification in municipal wastewater treatment system and disinfection at tertiary treatment but no assessment on the relationship between these two important pollutants from this source has been made.

Another important member of the microbial community is protozoan which have been observed to be responsible for reduction of particulate matter (suspended matter and bacteria) in wastewater treatment plant by its phagocytic and pinocytic modes of nutrition. Their predatory behaviour is selective with respect to structure of microorganism but not to type or functional group in the wastewater treatment

system. It is important to assess the effects their presence has on both biological processes to properly evaluate the link between both processes.

Nitrification results in the presence of nitrogenous oxides in the wastewater treatment system produced by the action of ammonia oxidising bacteria and nitrite oxidising bacteria respectively. These products are transient in steady conditions and should just be converted to the next product, but this does not always happen as a result of the differential sensitivities of AOB and NOB to DO, pH and temperature. Much more, different species of each type of nitrifying bacteria respond differently to different concentrations of substrate e.g for NOB in response to nitrite, *Nitrospira* has high affinity but low growth rate (k-strategist) while *Nitrobacter* has low affinity but high growth rate (r strategist). The availability of each type of nitrifier is therefore not certain and this makes nitrification an unstable process. It is therefore possible to have nitrite in the wastewater system. This product and its derivatives have been observed to be toxic to bacteria aquatic systems. However, though a lot of research has been carried out on its effects on the ammonia oxidising bacteria, up to date none has been done on its effects on the pathogens especially bacteria pathogens in the system. Disinfection of treated municipal wastewater is always at tertiary stage (Hwang 2010) with the use of chemical disinfectants and UV light which is very expensive. It is important to assess if the presence of nitrite could be responsible for pathogen reduction hence disinfection in nitrifying systems at secondary treatment.

The above three main points substantiate the aims for this research and in the next chapter the experimental plan and methodology will be discussed.

Chapter 4. General Experimental Design and Methodology

4.1 Theory:

The input of wastewater into bioreactor in a batch process suggest that the amount of organic matter and nutrients available for microorganisms is limited. A gradient of organic matter used as food is created in the reactor with detention time (figure 4.1). These limited conditions of food (organic matter) and nutrients results in a change in conditions of living for organisms so that they aggregate together in the formation of sludge floc as time increases. These changes are represented graphically in figure 4.2. This research is therefore designed to investigate the effect that these changes have on quantities of faecal coliforms numbers in the water phase.

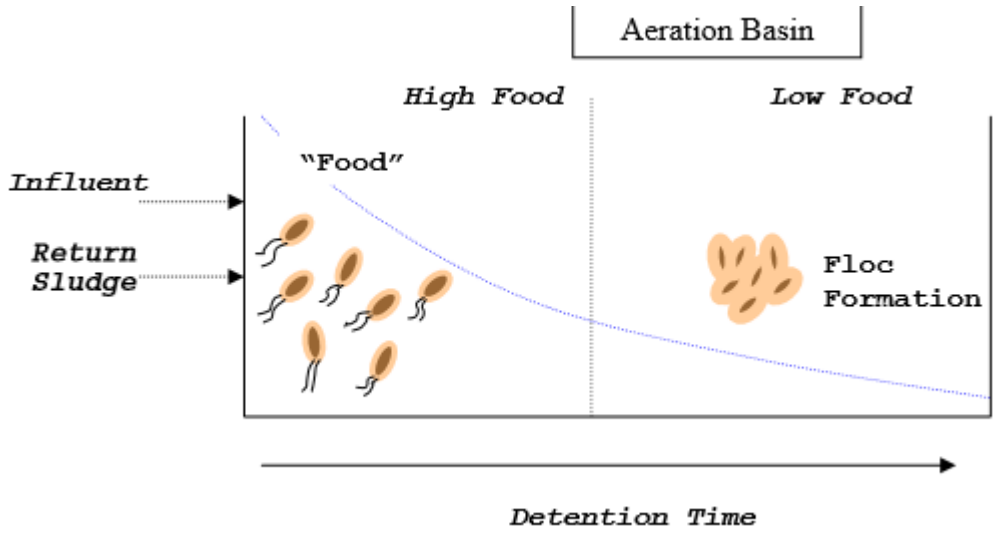


Figure 4-1: Aeration basin activity with time resulting in change in bacteria form and function as food reduces (adapted from Glymph 2013)

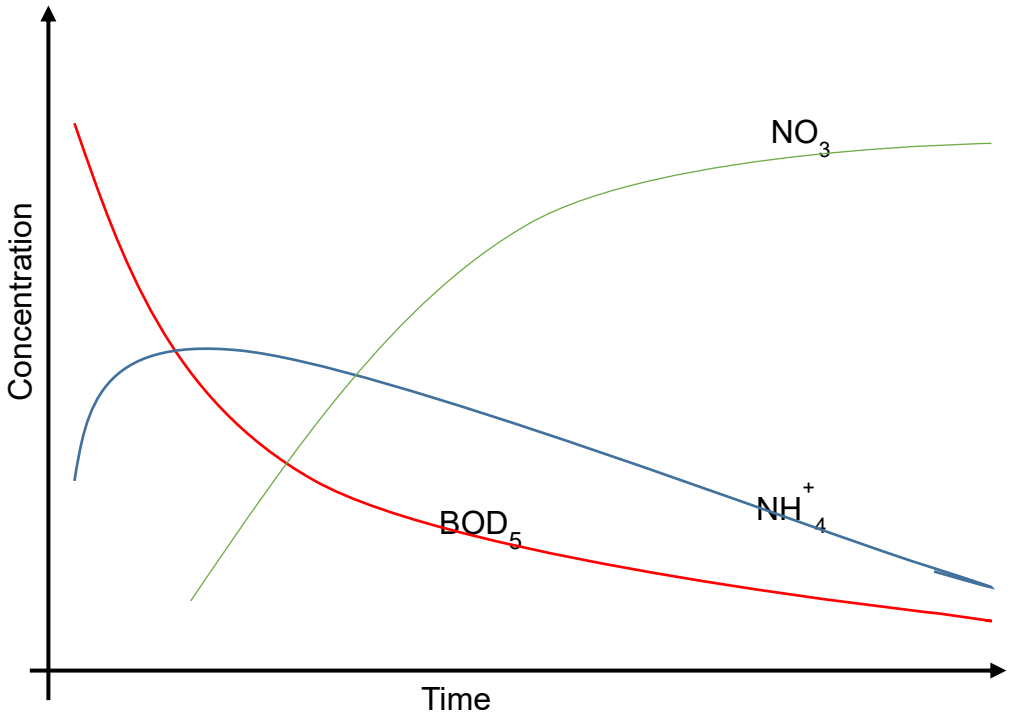


Figure 4-2 Graphical representation of the change in wastewater constituents as a result of nitrification

4.2 Experimental design

The focus of this research is to assess the relationships between the biological processes of nitrification and pathogen reduction at secondary treatment, so it is imperative to identify the factors affecting their occurrence and use these factors to investigate possible links between them. In view of this, the research objectives were assessed by means of experiment and observations were related to already existing scientific knowledge so that aspects of the investigation could be repeated or falsified as in the post positivist research paradigm (Burns 2000). The observation of change in concentration of substrates and products in biological reactions has been used to assess biological degradation (Cui *et al* 2014; Tang and Chen 2015; Tran *et al* 2014) in nitrifying systems already. Quantitative research methods were used to design laboratory batch experiments in an activated sludge reactor for observations which could provide answers to research questions. Details of the experimental

methodology are represented in figure 4.3. As indicated in the conceptual framework, (figure 1.0) three main studies, differently colour coded were carried out to investigate the objectives mentioned in section 1.2 and experimental methods used to investigate them are specified in figure 4.3 as well. However, the details of material and analyses are discussed in the subsequent sections.

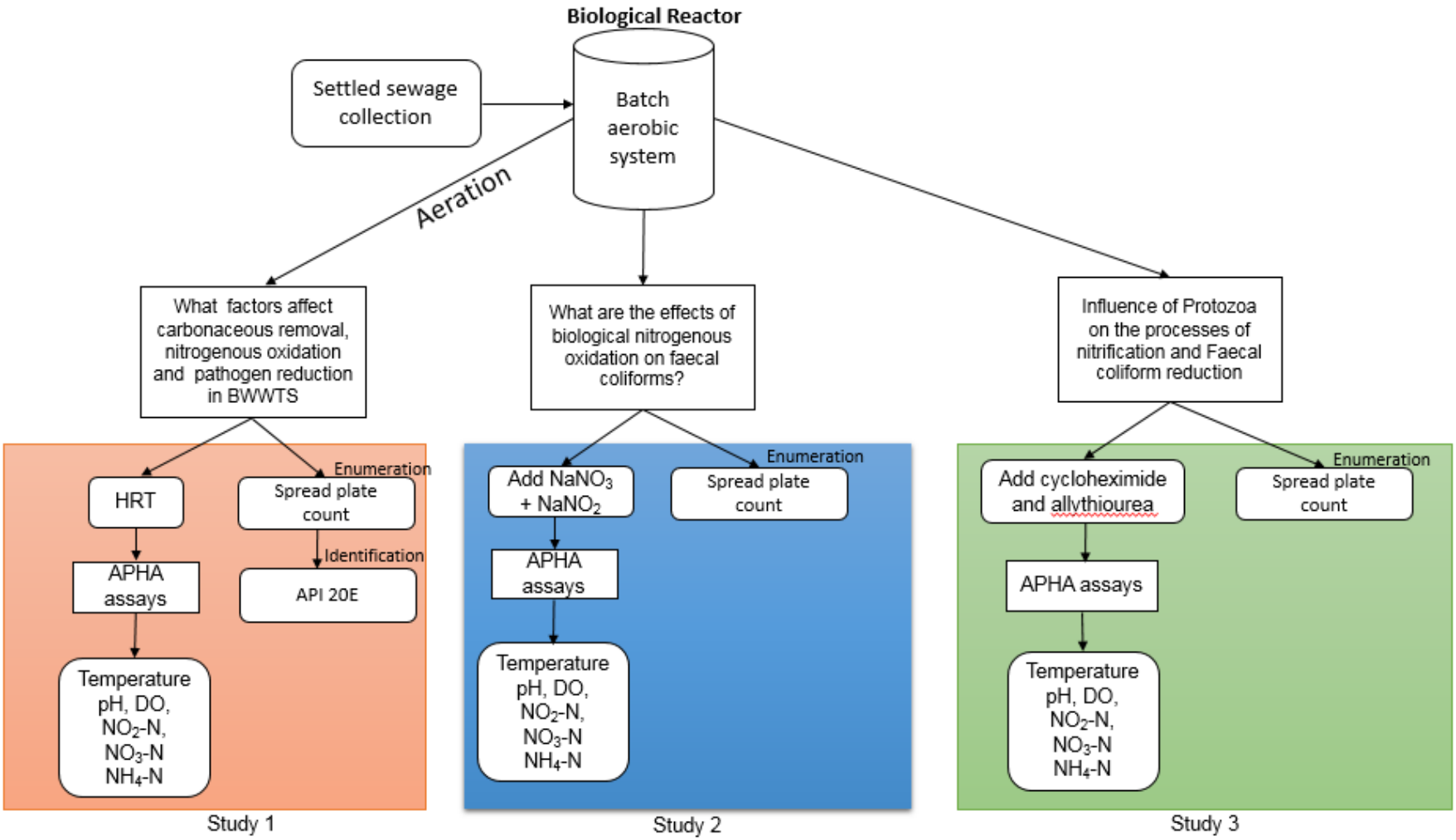


Figure 4-3 Schematic representation of Experimental Design and Methodology

(APHA 2005: American Public Health Association's standard methods of examination of waters and wastewater's assays)

4.3 Materials and Methods

4.3.1 Sample collection and preparation

Settled sewage obtained from primary clarifier and mixed liquor samples of aeration tank were obtained prepacked in 1L polystyrene bottles (figures 4.2 and 4.3) from Hatton wastewater treatment plant laboratory and transported to university laboratory. Hatton wastewater treatment is a full scale activated sludge, non-nitrifying plant, treating municipal wastewater. The use of fresh activated sludge in nitrification analysis is common (Campos *et al.* 2002; Juliastuti *et al.* 2003) and useful as it is less time consuming since organisms are active and don't have to get to steady state as occurs when pure cultures or nitrifying enrichments (Hu *et al.* 2004) are used. Also, information derived by their use is more kinetically and ecologically relevant as they represent real environmental samples than with pure cultures or nitrifying enrichments (Lee *et al.* 2015). Hence the quantities of AOB and NOB are not under contention as both types of organisms were subjected to identical environmental conditions.

It is recommended that wastewater for bacteriological studies be cooled preserved to limit the effect of environmental change on the bacteria activity and to prevent degradation of pollutants (Artiola et al 2004; Makela *et al*. 1996) but this was omitted to allow for water temperature rise necessary for nitrification activity. Very low temperatures would affect enzyme activities and growth rates of microorganisms particularly nitrifiers (Yao *et al.* 2013). Therefore, activated sludge bioreactor's performance would be greatly affected by low temperature hence a need to allow for increase in temperature. Reddy *et al.* (2017) used heat exchangers to increase ambient temperature of wastewater to about 20±2°C while industrially this temperature limiting situation is countered by bioaugmentation with other nitrifier products (e.g NitriStar 750) added to wastewater (Cui *et al.* 2014) to enhance nitrification activity.

On arrival at the laboratory the samples were kept on worktop to attain ambient temperatures of between 17 to 20°C which were within range permissible for nitrification (Metcalf and Eddy 2003). The steading of waste samples for temperature rise also allowed for mixed liquor samples to separate such that suspended solids settled and are concentrated at bottom of bottles. Thereafter, the clear supernatant was decanted (Tang and Chen 2015) to concentrate sludge for use as return

activated sludge. This concentrate mixed liquor is mixed with settled sewage (cloudy) (figure 4.5) to make a working volume of 3 L for aeration.

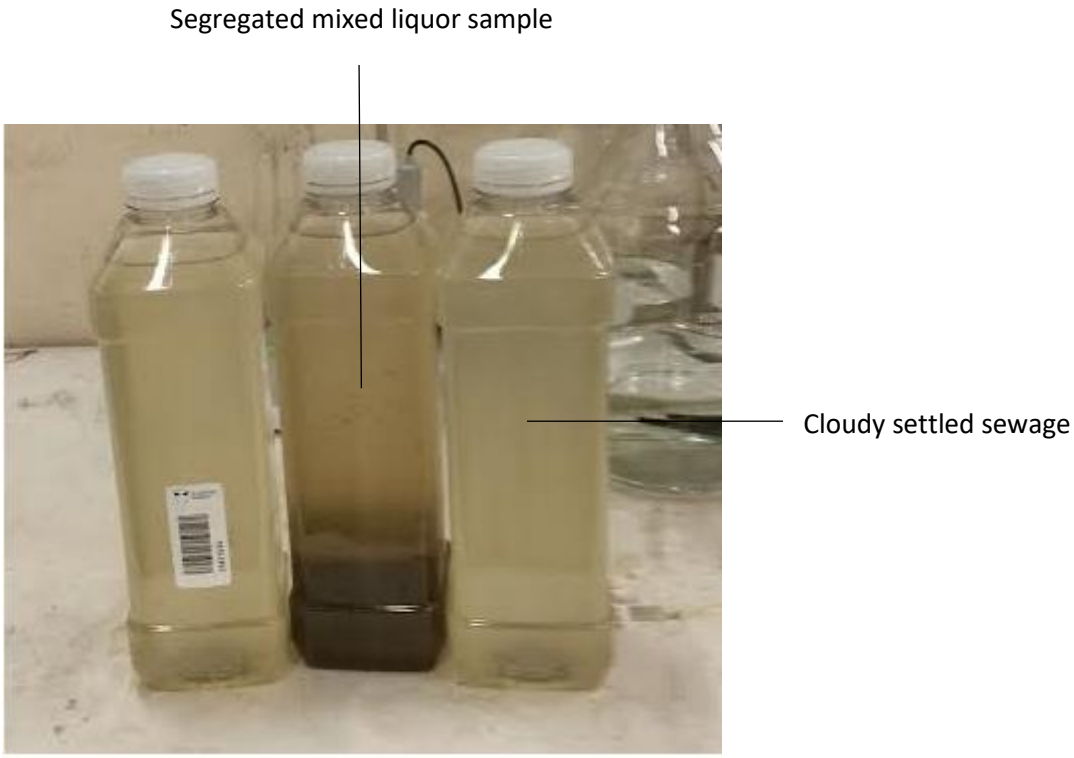


Figure 4-4 Samples obtained in 1 L polyethylene bottles

4.3.2 Batch reactor

Experiments were conducted with the use of a laboratory scale activated sludge batch reactor constructed by laboratory technicians as illustrated in figure 4.4. Batch reactors are said to be useful in the study of populations of microorganisms and the reactions involved in nutrient recycling because nutrients can be recycled within the microcosm thereby self-sustaining the closed system for a short time (Petropoulos and Gilbride 2005).

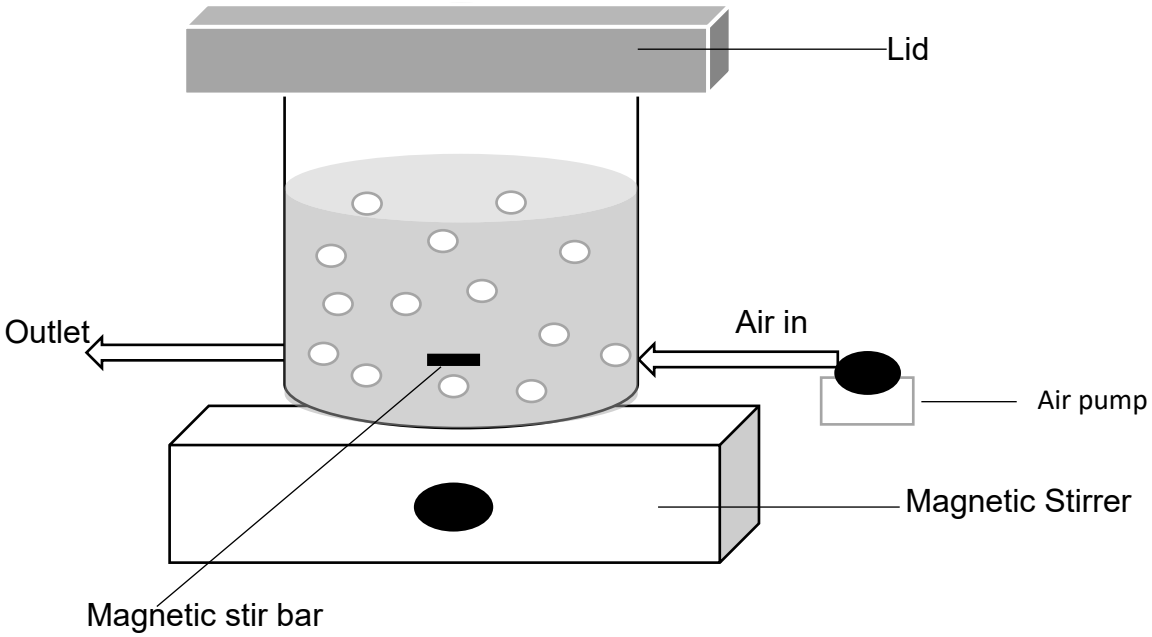


Figure 4-5 Schematic representation of laboratory scale batch reactor use for experiment

Size of reactor was about 7 L and wastewater ready for aeration was poured in batches through the top. The lid was kept over the reactor continuously throughout aeration period to limit loss of water by evaporation but was only opened for the collection of samples for analysis. For each run, a new sample was obtained from the Hatton plant and aeration was continuous through the run. Analysis each sample were done in triplicate each run. However, one grab sample was used for all analysis at each each interval and this was collected from the reactor with the use of a 50 mL beaker each period of analysis during runs. During each run of an experiment

analysis were carried out at several intervals. The number of experimental runs, interval for analysis and type of analysis depended on the specific experiment.

4.3.3 Batch Extended Aeration Treatment

At temperature above 17°C, the mixture was put into reactor and aeration started immediately. A lid was placed at top of aerator to prevent evaporation which could lead to dehydration (figure 4.4). Complete mixed aeration with the purpose of making available oxygen for aerobic processes and stirring wastewater was achieved (Qasim 2017) by use of Elite 801 air pump (Rolfe and Hagen) which supplies air as rising bubbles. These bubbles enhance oxygen transfer as their varying sizes increase surface area for contact of oxygen with wastewater (Roman and Muresan 2014) and the vibrations caused by the pumping of air mixes wastewater steadily. Diffuse aeration systems with small fine bubbles are said to be most efficient in oxygen transfer in activated sludge system as they supply a lot of air at low pressure and their small sizes provide increase surface area per unit volume implying more oxygen transfer (Boyd and Moore 1993). However, they are not as cost effective as the air pumps used here (Roman and Muresan 2015).

Mixing was enhanced using a magnetic stir bar and magnetic stirrer placed at the bottom of the reactor as was done by Melcer (2003). The magnetic stir bar was controlled by magnetic field from magnetic stirrer device beneath. More stirring occurred when grab samples were obtained with the use of a manual stirrer to ensure sample obtained was an exact representative of whole sample in reactor.

After grabbing sample was filtered by gravity through 8 µL Fisherbrand grade 601 cellulose general-purpose filter paper (Fisher Scientific, UK) which are recommended for filtration of samples for environmental analytical work and microbiological analysis (Fisher Scientific 2018). Wastewater treatment period and type of analysis performed, varied with respect to the objective of the study under consideration at the time. In the next section a look at basic analysis that were required for all studies is carried out.

4.3.4 Assessing Carbonaceous and Nitrogenous oxidation

The concentration of organic carbon as food for heterotrophic bacteria reduces with time as observed in figure 4.1 and 4.2. This process is biological oxidation of organic carbon and is measured as carbonaceous biological oxygen demand (cBOD)

implying that, organic carbon oxidation is the process assessed here and dissolved oxygen concentration is the substrate measured. Nitrogenous oxidation which succeeds carbonaceous matter reduction in aerobic systems is assessed by measuring concentrations of substrate (ammonium) and product, (nitrite and nitrate) as was done by previous studies (Coskuner and Jassim 2009; Kumari *et al.* 2011; Tang and Chen 2014). These concentrations as well as changes in COD and BOD_5 were carried out to understand the effect of oxidation on carbonaceous and nitrogenous pollutants. Other researchers have assessed nitrification by using molecular methods to estimate the ratio of nitrifying organism to heterotrophic bacteria population so that change in ammonia monooxygenase (amoA) and 16s RNA gene ratio throughout the treatment time through time was assessed (Mertoglu 2008) but the drawback to this method is the high cost. Another method of assessment of nitrification could be by measuring the carbon dioxide uptake in nitrifying reactors (Beccari *et al.* 1979; Blackburn *et al.* 2007) but this method could not be applicable as samples were obtained from a non-nitrifying plant.

4.3.5 Estimating Pathogen reduction

The change in pathogen numbers was estimated by assessing change in quantities of faecal coliforms and *E. coli* as biological oxidation processes taking place in the activated sludge reactor. These organisms were chosen because they are standard indicators of faecal pollution (Ashbolt *et al.* 2001) and had also been widely used in pathogen identification research (Bahrim *et al.* 2012).

Cultivable methods were used to facilitate detection for enumeration, and these are important as detection of only viable bacteria is obtained. However, only small quantities of sample (Koster *et al.* 2003) are used to represent the whole lot so that identification with cultivable methods are not fully representative. In activated sludge systems where cells are kept in suspension by water movement, Venter *et al.* (2000) stated that plate counts may not truly represent the composition of the population as these movement disrupt sludge floc so that viable single cells may not be yielded uniformly. This problem is countered by shaking the filtrate so that an approximate representative of the whole sample could be derived from the small sample used. Molecular methods, which are said to be sensitive and reliable however (Venter *et al.* 2000), identify dead and even inactivate bacteria in their assessments, which are not relevant for this study and are expensive.

The heterotrophic plate count (standard) (APHA 1999) was used for enumeration and specifically, the viable spread plate method with the use of HiCrome coliform agar (Merck, UK) recommended for the simultaneous detection of *E. coli* and total coliforms in water samples (Manafi and Kneifel 1989). This method is widely used as it is proven to present better and more consistent results when compared with different bacteria enumeration methods like most probable number, petrifilm disposal plate method, membrane filtration, standard pour plate and substrate technology methods (Wohlsen 2006). The spread plate technique is also said to support the growth of aerobic organisms as organisms grow on the surface but is limited by being able to analyse only very small samples (0.1-0.2 mL) (Koster *et al.* 2003). The growth of colonies on surface of media makes it easy for transfer and isolation than with pour plate where they are submerged (AHPA 1991). It is also easier to differentiate colonies with spread plate method than the pour plate method but scoring of colonies is not easy. More so, spreading is done on relatively cold media to prevent any effects of heat shock on organisms as occurs with pour plate method (AHPA 1999).

The HiCrome coliform agar (peptone special 3 g, sodium chloride 5g, dipotassium hydrogen sulphate 3 g, potassium dihydrogen sulphate 1.7 g, sodium pyruvate 1 g, L-tryptophan 1 g, sodium laurly sulphate 0.1, chromogenic dye mixture 0.2 g and agar 12 g) is a chromogenic media whose activity is based on the presence of enzymes β-galactosidase and β-glucoronidase in the sample (Merck 2018). These enzymes are produced by coliforms and *E. coli* respectively, breakdown substrates that are found in the chromogenic mixture (Himedia 2018) thereby producing distinct colour indicative of specific bacteria colonies (Akter *et al.* 2014) present. β-galactosidase breaks down idoxyl-β-galactoside chromogen resulting in reddish coloration (Salmon red) of coliform colonies while β-glucoronidase breaks down the idoxyl-β-glucoronide and produces a dark blue/violet coloration (Merck 2018). However, β-galactosidase is also found in other microorganisms which are present in wastewater samples like *Vibrionaceae*, *Pseudomonaceae*, *Neisseriaceae*, yeast, protozoa and fungi (Koster *et al.* 2003). Also, some strains of *E. coli* e.g*. E. coli 0157* don't grow at 44°C and are β-glucoronidase negative hence cannot be identified by this method (Koster *et al.* 2003). *Yersinia*, *Shigella*, *Salmonella*, *Citrobacter*, *Edwardia* and *Hafnia* strains are also said to produce β-gluconidase hence there is a

need to confirm colonies as *E. coli* to avoid false identification (Koster *et al.* 2003). Also, previous research indicates that enzyme base media activities are usually disrupted by other bacteria (Koster *et al.* 2003).

In the media, growth nutrients are supplied by peptone special and sodium pyruvate while the phosphate buffers the medium. Sodium lauryl sulphate helps to inhibit the growth of Gram-positive bacteria and novobiocin is added when high numbers of Gram-positive organisms are expected like with the use of wastewater samples of mixed population, to reduce competition for nutrients. The presence tryptophan is required to increase the activity of indole so that the detection of organisms in the combined chromogens increases (Himedia 2015).

4.3.6 Bacteria identification

In order to identify the impact of treatment on the quality of water, after enumeration to estimate reduction, isolation and identification of faecal coliform species and *E. coli* was carried out. Isolates were taken from the growth culture on HiCrome agar plates and pure cultures were grown on nutrient agar (Thermo Fisher Scientific, UK) plates. Nutrient agar is made up of lab-lemco powder 1 g/L, yeast extract 2 g/L, peptone 5 g/L, sodium chloride 5 g/L and agar 15.0 g/L (Thermo scientific 2018). *E. coli* and faecal coliform colonies where distinguished by their colour on chromogenic media which was dark blue and pink respectively. Though *E. coli* on HiCrome media was distinctinguished by dark blue coloration as a result of reaction of β-glucoronidase as mentioned in section 4.1.2 above, other organisms can produce β-gluconidase so that the presence of *E. coli* needs to be confirmed. This was done by a spot test with Kovac's reagent (contains p-dimethylaminobenzaldehyde, isoamyl alcohol and hydrochloric acid). An indole test in which tryptophanase splits tryptophane into indole, pyruvate and NH_3 so that the p-dimethylaminobenzaldehyde then reacts with indole forming a cherry red coloration (Jvo 2009).

Identities of specific faecal coliforms were obtained with the use of the analytical profile index kit (API 20E) (Biomerieux USA) which contained twenty-one biochemical tests and a data base standardized for the identification of *Enterobacteriaceae* and non-fastidious gram-negative bacteria (Biomerieux 2002). The kit was made of dehydrated substrates in microtubles which when inoculated with bacteria suspensions resulted in metabolism which produced colorations after

the addition of specific reagent as indicated in instruction manual. In principle, during the fermentation of carbohydrates (contained in the reagents) the pH within the cupule changes and is shown indicators therein. Bacteria grow if they can utilize the substrate in tubule and a positive result is indicated by growth. A reading table was provided to enable reading of the reactions and identification of specific faecal coliform was obtained by using analytical profile index data base (Biomerieux 2002).

According to (Juang and Morgan 2001) API 20E method is limiting as it can only be used to identify gram negative bacteria only to genus level in activated sludge systems but Venter *et al.* (1989) believe that they provide reliable result for activated sludge systems as long as fermentation test are checked after 24 hrs and by supplementing the API 20E oxidase test with a standard oxidase test. API test have been used in wastewater research in the identification of bacteria in petrochemical wastewater (Rojas *et al.* 2007) and in activated sludge systems (Hart and Melmed 1982; Buchan 1983; Lotter and Murphy 1985; Kerdarchi and Harley 1987). Here, they were used to assess bacteria character in relation to phosphate removal hence they could be applied when nitrogen is applicable.

However, API 20E is normally used for the routine identification of bacteria in environmental studies, as they are of low cost, easy to use and with short response times. For critical research work molecular identification methods based on cloning and sequencing of genetic material like denaturant gradient gel electrophoresis, fluorescence *in situ* hybridization are used (Sanz and Kochling 2006). Though these are capable of identification to specie level they are very expensive, have long response times and require high skilled labour.

4.3.7 General Analysis

A combination of spectrometry and multivariate analysis is being used more frequently in the recent times to monitor wastewater physico chemical parameters as they have the advantage of not needing reagents or solvents, are usable *in situ*, online or anywhere (Mesquita *et al.* 2017). Biological wastewater treatment processes have made use of Ultraviolet visible (UV-Vis), infrared (IR) and fluorescence (FLC) spectroscopic methods. In UV- Vis method, sample and radiation interact between wavelength range of 200-780nm (Lourenco *et al.* 2012). These spectroscopic methods are limited by their sensitivity to temperature and

environmental factors, depend on online methods and are mainly used in laboratories (Mesquito *et al.* 2017). They don't detect saturate hydrocarbons or sugars and could be affected by the presence of suspended particles which scatter light. However, there are methods are easy to follow and provide results quickly.

The filtrate prepared above was used for analyses of both physico-chemical and microbiological wastewater characteristics. These included five-day biochemical oxygen demand ($BOD_{5)}$, ammonium nitrogen (NH_4^+-N), nitrate nitrogen (NO_3^--N), nitrite nitrogen (NO_2^--N), and chemical oxygen demand (COD), as well as faecal coliform and *E. coli* numbers respectively. Faecal coliform and *E. coli* colonies were also identified after enumeration on cultured plates. Physical characteristics like pH, temperature and dissolved oxygen were measured as well.

Standard methods of analysis of wastewater (APHA 1999) were used to analyse for wastewater characteristics to assess changes in quantities of physico-chemical and microbiological characteristics in the system as reaction proceeded. First nitrification was established in the system and this was done by aerating wastewater for four hour, three days and five days. And testing for change in wastewater nitrification indicators ammonium nitrate, nitrate nitrogen and nitrite nitrogen.

4.3.7.1 Physio chemical Analysis

Factory prepacked reagents of Hach laboratory were used for chemical analysis and values of wastewater characteristics were obtained with help of DR5000 UV-Vis spectrophotometer. Each test kit was supplied with instructions and results were guaranteed accurate if adhered to precisely (Hach 2013). This is because Hach reagents come premeasured in sealed cuvettes making them safe, error free, easy to use and less time consuming as no pipetting or contact with chemicals was necessary. Cuvettes used were made of glass which facilitated observation of colour changes during reaction. The integrated barcodes on glass of cuvette enabled the spectrophotometer, with the help of the integrated barcode reader, to identify the specific test and obtain 10 measurements as cuvette rotated and automatically generated a final value eliminating outliers with the use of the reference beam technology. This ensured reliability of results and reliability was also enhanced by using factory supplied standards for each test with spectrophotometer (Hach 2013). The principles underlining specific analyses are hereby discussed.

4.3.7.1.1 Chemical Oxygen Demand (COD)

In the operation of wastewater treatment plant and the characterisation for water quality worldwide, COD is an important parameter used to determine organic load based on complete oxidation of organic load to carbon dioxide and water by reaction with potassium dichromate ($K_2Cr_2O_7$) in accordance to ISO15705 (APHA 1999). A measure of dichromate is given the amount of which is the international standard (APHA 2003). It is measured as a complementary test for BOD_5 since it considers both organic and inorganic oxidation in the wastewater sample but has the advantage of shorter time hence is more often useful for process monitoring and control (Hach 2016).

The colorimetric method was carried out with use of Hach LCK 614 kit in which the chemically oxidizable substance in sample reacts with sulphuric acid-potassium dichromate solution in the presence of silver sulphate catalyst for 2hrs. A yellow greenish coloration of chromate ion (Cr^{3+}) is produced and its spectrophotometric evaluation at 620nm wavelength gives the value of the amount of chemical oxygen demand in the sample. The silver sulphate catalyst enhances the oxidising ability of recalcitrant compounds (Kolb *et al.* 2017) and suppresses any interference (Hach 2016). Municipal wastewaters are rich in organic matter and are expected to have COD values higher than 50 mg/L for which this method is recommended (AHPA 1990). However high values of municipal wastewater COD could be caused by the presence of high quantities of chloride which are oxidizable by Cr (VI) (Kolb *et al.* 2017). Increase of COD could also result from the presence of nitrite which causes an increase of 1.1 mg O_2/mg NO_2-N but because nitrite concentrations hardly exceeds1-2 mg/L in wastewater treatment steady systems impact of nitrite is minimal (APHA 1999).

Though a quick and reliable method, interference from suspended matter or turbidity will hinder analysis. However, this problem, which iapplies to all spectrophotometric reading with cuvettes, was minimised by filtration procedure. A drawback of this method is its generation of hazardous waste like mercury, silver and hexavalent chromium. These are safely disposed of by the product supplier (Hach 2013). More so, the use of small quantities of sample as required by Hach procedures limit the amount of waste produced (APHA 1999).

COD could also be obtained by the titrimetric method which is less expensive but labour intensive and prone to error as measurements of reactants are necessary as opposed to premeasured reagent with the Hach cuvettes (APHA 1999). Other wastewater COD analysis methods such as the wet oxidation using manganese and silver nitrate are being tried (Kolb *et al.* 2017).

4.3.7.1.2 Ammonium Nitrogen (NH_4^+-N)

Organic nitrogen is gradually converted by ammonification to ammoniacal nitrogen, which is unstable as it easily converts to ammonia (equation 4.1) depending on pH (Walker and Simon 2012).

$$NH_4^+ \rightleftharpoons NH_3 + H^+ \quad (4.1)$$

Ammonium nitrogen is a measure of the amount of ammonia and an indicator of the presence of untreated sewage in the treatment system (Walker and Simon 2012). A colorimetric method, using the Hack LCK 303 cuvettes of range 2 to 47 mg/l NH_4^+-N, which is within the range expected for municipal wastewater, was used here. Cuvettes stored in fridge was brought out and allowed to acquire room temperature before analysis commenced. Sample was filtered and procedure followed as directed on the manual so that ammonium ions in the sample comes in contact and reacts with hypochlorite and salicylate ions in the presence of sodium nitroprusside catalyst forming indophenol blue coloration (APHA 2014). The intensity of colour formed is directly proportional to the concentration of ammonia in the sample and this is read at about 690nm wavelength of the spectrophotometer. Maximum concentration is obtained after a reaction time of 15 mins and value remains constant for 15 mins as the process is time dependent. PH of sample should be between 4-9 and temperature around 20^{o}C (Hach 2000). Though quick, method produces hazardous waste is produced. Also, procedure only applies to a specific concentration range (an issue with hach cuvette test) implying that new kits are required for different concentrations making it expensive (Hach 2014).

Ammonium nitrogen concentration could also have been measured with the use of the electrochemical or Ion selective electrode in which ammonium ions which converted to ammonia gas which causes a proportional change in pH and gives the concentration of the ammonium (Hach 2014a). This method is quite popular, said to be cheap and accommodates a wide range of ammonia concentrations but it is time

and labour intensive, requires expertise to handle electrode and membrane as well as is very difficult to use at concentration below 0.1-1 mg/L NH_4^+-N. So ever due to time constraints and number of test the method above was used in this study.

4.3.7.1.3 Nitrite Nitrogen (NO_2^--N)

Nitrite nitrogen is the intermediate product of different wastewater processes; nitrification (equation 4.2), denitrification and nitrate reduction to ammonium (Davis *et al*. 2000).

$$NH_3 \leftrightarrow NO_2^- \leftrightarrow NO_3^- \qquad (4.2)$$

It is very transient and in steady conditions, is present in minute quantities of 0.2 to1 mg/L as they are readily oxidised to nitrates.

Nitrite nitrogen was determined by the Griesse method (Nagaraja *et al.* 1998) in which sulphanilamide, an amine, undergoes a diazotization reaction with nitrite of the sample in the presence of an acid to form an intermediary diazonium salt (Hach 2001). This salt in turn reacts with an aromatic amine, napthyl-1-amine, to form a deep yellowy orange, azo dye (Moldovan 2010). The Hack LCK 341 kit was used here and the colour was directly proportional to the amount of nitrite in the sample at measurement wavelength 515 nm (Hach 2001). This procedure could be affected by the presence of chromium (VI) or copper (III) ions and is the most common method as the procedure is easy to follow, short response time and offers high sensitivity (Davis *et al.* 2000; Moldovan 2012) but its short range of applicability is a disadvantage (Qader 2013).

Due to nitrite toxicity, there are several methods for determining nitrite in water and wastewater samples including chromatography, polarography, flow injection analysis and voltammetry. However, these methods have drawback of long response times, require expensive instruments, which in turn require expert technicians and are less sensitive that the photometric methods (Moldovan 2012).

4.3.7.1.4 Nitrate Nitrogen (NO_3^--N)

Nitrate is the most complete oxidised form of nitrogen formed by the biodegradation of ammonia. Its presence in wastewater is an indication of biological waste at stabilized state or the presence fertilizers from farm waste (Henze *et al.* 2001).

Therefore, its assessment reveals the degree of oxidation in the biological treatment plant.

A colorimetric analysis with the Hach LCK 339 kit was used. Nitrate ion in the sample, react with 2-3-dimethyphenyl ions in the presence of sulphuric acid and phosphoric acid to form orange coloured 4-nitro 2.6-dimethyphenyl ions. This colour is quantified by spectrophotometry at 320 nm wavelengths to give the quantity of nitrate in the sample (Hach 2005). This method is said to be subject to interferences due to turbidity resulting from the concentration of oxidizable substances. However, this interference is minimised by diluting wastewater with dilution water 50:50. This could also be resolved by filtering the sample with a 0.45-micron filter paper. Advisable used for substances with COD less than 500 mg/L. Another interference to result could be nitrite concentration greater than 2 mg/L. The 15 mins reaction time for full colour and temperature at 20-25^{o}C are important to achieve good results.

The advantage of this method is the quick response time of 15mins due to fact that all reagents are already measured in cuvette hence less labour required as reliable results are obtained if procedures in manual are adhered to. However, the range of usage is limited, and this adds to the cost. Other methods of determination of nitrate such as cadmium reduction method, the nitrate electrode method and the chromatographic method are available (APHA 1999) but their main drawback is the long response times and they require experience labour.

4.3.7.1.5 pH and Temperature

Disinfection processes are pH dependent (APHA 1999). Direct measurement of pH is important as pH varies as temperature, water movement and changes in chemical content due to biological and chemical processes occur. There is need for simultaneous and continuous measurements of pH and temperature. The Hach Sension 3 uses the USEPA electrode method 8156 and is made of an electrode containing silver in a potassium chloride electrolyte solution. The concentration of hydrogen ions (H^{+}) in the solution, induces voltage changes and these voltage changes vary with the pH of the sample at the glass liquid interphase when the probe is inserted into the liquid (Hach 2013). Calibration of metre was done before immersion in sample and by immersing probe into pH 4, pH 7 and pH 10 standard solutions. pH measurements obtained by this method have an accuracy of ±0.01

(Hach 2000). This method is said to be very sensitive and most commonly used but the drawback is the high initial cost and care that is required of glass electrode. Optional method of measuring pH is with the use of pH test strips made with indicator solutions of organic pigments. However, with this pH is a just an estimate (Haggins 2014).

4.3.7.1.6 Dissolved oxygen (DO)

The amount of gaseous oxygen dissolved in water (DO) was measured with the use of the Hach high quality digital (HQD) 40d luminescent dissolved oxygen (LDO) probe (Hach Lange GmbH) method 10360 which is USEPA approved (Hach 2015). The value obtained by this apparatus is an indicator of the water's ability to support life and therefore essential to ensure survival of biological treatment biota (Hach 2004) as mention in section 2.2.4.2 above. The probe of this metre was fully inserted into wastewater in reactor which was being stirred by magnetic stirrer. This probe has a clear but oxygen impermeable substrate capable of recognising automatically the presence of oxygen. This is due to the presence of an oxygen sensitive luminescent dye and a scattering agent. Oxygen presence gives a blue light and when the dye is exposed to this, it in turn gives a red light which is scattered by the scattering agent in the sensor matrix making the sensor impermeable at moment. Red light generates pulses, which are used as internal references, and the duration of the luminescence is proportionate to the concentration of dissolve oxygen in the sample, which is read on the display (Hach 2015b). The probe of DO meter is easy to manage, as calibration is factory done and it requires no chemical addition (Jounneau *et al.* 2014). Much more, oxygen is not consumed by sensor and probe readings are not affected by water movement or any electrochemical fields (Klimant *et al.* 1995).

This automated method is easy and quick to use. The meter is factory calibrated and can hold calibration for several months (Hach 2015b), but maintenance and adhering to procedures in manual are important to achieve adequate results. This apparatus could also be used for online monitoring. The drawback however is high initial cost. Other methods of measuring dissolve oxygen like the Wrinker's method could not be use as interference from nitrite will be a concern and the partial oxidation of organic matter due to the presence of oxidized manganese ions will result in negative errors (APHA 1999). This method is also more time consuming and labour intensive than

the optical probe method above. Another method is the electrochemical probe, which is cheaper than the optical probe and with less response times. However, due to oxygen penetration through the membrane a build-up of oxidised material on either the anode or electrolyte would result in non-functionality of the probe. This imples a high maintenance cost for probe is required (Li *et al.* 2015).

4.3.7.1.7 Biological Oxygen Demand (BOD)

BOD is defined as the measure of oxygen removed from wastewater by aerobic heterotrophic organisms as they use up organic matter for growth and metabolism (Brookmann 1997). It is an indication of the amount of molecular oxygen used during the respiration of microorganisms growing on organic matter in a wastewater sample at a 20°C for 5-days (Jouanneau *et al.* 2015). 5-day BOD (BOD_5) is standard derived from the fact that the longest time it took for the Thames river to travel from source to estuary was 5-days and this was enough to satisfy requirement for river quality (Changrekar and Kharagphur nd; Great Britain 1908). This gives a measure of the biodegradable pollution of water (Nagel *et al.* 1992), which can be applicable in estimating the fraction of effluent which is biodegradable from BOD_5/COD ratio. The wastewater treatment and discharge conformity with respects to regulation and the size of treatment plant for a location could therefore be obtained by deriving the COD/BOD_5 ratio (Jouanneau *et al.* 2015).

A manual close bottle test was carried out here. Dilution water needed in this experiment was prepared by the addition of magnesium sulphate, calcium chloride, ferric chloride solutions as well as potassium phosphate buffer, to distilled water in a concentration of 1 ml/L each, to provide osmotic balance, buffer the pH and provide essential nutrients which support growth of bacteria. This water was subjected to aeration by gently bubbling of air for at least six hours (AHPA 1998) to make it fully saturated with oxygen necessary for the aerobic process of biodegradation of organic matter by microorganisms. Specific quantities of wastewater obtained from grab sample (seed) were put into 275 mL labelled BOD bottles. For each sample, three different sample volumes were used for same size BOD bottle and these were done to provide an over lapping range in expected BOD_5 concentrations (Expected BOD_5 range being 230-560 mg/L) (Henze and Comeau 2008). 2 mL, 4 mL and 6 mL of each sample sample analysed on day 0 and 5 mL, 10 mL and 15 mL of each treated sample along the treatment time during run. And into each of these bottles 1

mL allylthiourea (2mg/L), which inhibits nitrification was added as well as dilution water to about three-quarter of the bottle.

The Hach high quality digital HQd (LDO) probe (Hach 2017) discussed in section 4.3.7.1.6 was used to measure the dissolve oxygen concentration in each bottle as stirring with magnetic stirrer occurred simultaneously to ensure uniform distribution of DO. Two other BOD bottles containing dilution water and 1 mL allylthiourea only and were set up as control and contained dilution water were put in using the procedure above. Initial DO values were obtained for each bottle after filling with distilled water to three quarters of bottle and bottles were completely filled up with dilution water and closed hermetically with stopper to ensure no vacuum left or no inlet of air so as to avoid escape or inflow of air hence prevent addition of oxygen or bacteria. All BOD bottles were thereafter transferred to an incubator where they were kept at 20^{o}C for five days. After five days the bottles were brought out of incubator and DO measurements taken again as final DO readings.

Calculations for BOD_5 were done using the following formula:

$BOD_5=\frac{(initial\ DO-final\ DO)\times BODbottle\ volume}{sample\ size}$ for each bottle and the average per sample was estimated in mg/L.

The above is the standard BOD procedure (ISO 5815 -1 2003), which in an aim to imitate the natural environment are based on the unknown microbial diversity or density (Jounneau *et al.* 2014). One drawback of the standard method is the 5 days' time constrain (Reidel *et al.* 2002) which does not meet industrial demand for quick assessment necessary for improvement of wastewater monitoring and therefore not practical for online monitoring which is required for the identification of sudden adverse operational or process conditions in the treatment process (Logan and Wagenseller 1993). Another issue is its variability between laboratories which is as a result of the variability in microbial population in water samples (Guyard 2010). Much more the biodegradability of organic matter is limited by oxygen concentration in the bottle (Jounneau *et al.* 2014) and the dilution involved reduces substrate as well as microorganism concentrations hence a reduction in reaction kinetics (Logan and Wagenseller 1993) which implies that the actual treatment conditions are not reflected. Further, one is not able to tell from this test if the change in BOD is as a

result of the toxic substances always present in wastewater and capable of inhibiting bacterial oxidation activity (Ademoroti 1985).

Other researchers assess BOD by measuring carbon dioxide produced during biodegradation process (Chiapini *et al.* 2010) but efficiency of system is very low (Jounneau *et al.* 2014). Photometric methods using the Macherey Nagel and Hach Lange 554 and 554 cuvettes could help in reduction of working space which is observed in the standard procedure (Jounneau *et al.* 2014). Also available is a manometric method which could be used with undiluted samples as the pressure reduction by oxygen consumption during organic matter biodegradation is measured rather than oxygen utilised as with Hach Lange BODtrak apparatus (Jounneau *et al.* 2014). For shorter analysis time and less space biosensory methods are available. Some based on bioluminescent bacteria eliminate the aspect of bacteria variability by assessing the functioning of a specific specie (Sakaghuchi *et al.* 2007). Other biosensors with redox mediators eliminate the limitation of oxygen by relating BOD to amount of biodegradable organic matter (Pasco *et al.* 2000; Nakaruma *et al.* 2007). However, BOD bio-sensory data is said to be irrelevant for environmental monitoring because natural environments are varied in both physico-chemical and biotic composition hence the continuous use of the standard procedure.

4.3.7.2 Bacteriological Analysis

In this section details of the assays involved in enumeration and identification are discussed.

4.3.7.1.8 Enumeration

In order to enumerate faecal coliforms and *E. coli* filtrate was put into 2 mL sterile Eppendorf tubes and stored at 4°C in refrigerator until culturing for four hours. These tubes have airtight lids so that liquid is kept tight in tube, cannot be spilled during transportation or contaminated internally or externally. Much more, tube is made of plastic material which enables it to resist fluctuations of temperature so that microorganisms in samples are not stressed by temperature changes (Eppendorf 2015).

HiCrome media was made by adding 13.5g of HiCrome agar powder (Sigma Aldrich, UK) to 500 mL of distilled water with the addition of 12.5 µL of novobiocin (Sigma Aldrich, UK) and the mixture agitated in 1L glass bottles and sterilized by autoclaving

at 121°C for 15 mins (as per manufacturer's instruction). Media was cooled in a water bath at 55°C and poured into petri dishes under aseptic conditions (plate close to Bunsen flame) and allowed to set at room temperature.

Ten-fold dilutions of sample were made in 0.9% NaCL, using bio pipettes and sterile pipette tips. This dilution process reduces the number of bacteria per unit volume so that observed is at countable level (PHE 2014). Also, Sodium chloride provides an isotonic environment, which prevents lysis of bacteria or growth so that actual number of bacteria in sample is preserved and cultured. 200 µL of 10^{-1}, 10^{-2}, 10^{-3} 10^{-4} and 10^{-5} dilutions each were transferred by means of pipette unto molten agar plates under aseptic conditions and a sterile glass spreader (spreader was sterilized by dipping into 70% alcohol and swiftly passing through blue flame of Bunsen burner each time) was used to spread out samples on surface. Spreading was done in triplicate per dilution and plate were immediately covered and left to on worktop for inoculum to set on surface of agar before transportation to incubator where they were kept for 24 hrs at 44.5°C. This temperature is important to distinguish FC from other bacteria of the total coliform group which grow on lactose containing media at media between 35-37°C (Vilanova *et al.* 2004). The presence of salmon red colonies indicating faecal coliforms, purple/violet colonies indicating *E. coli* and others which were creamy in colour was observed (Figure 4.6).

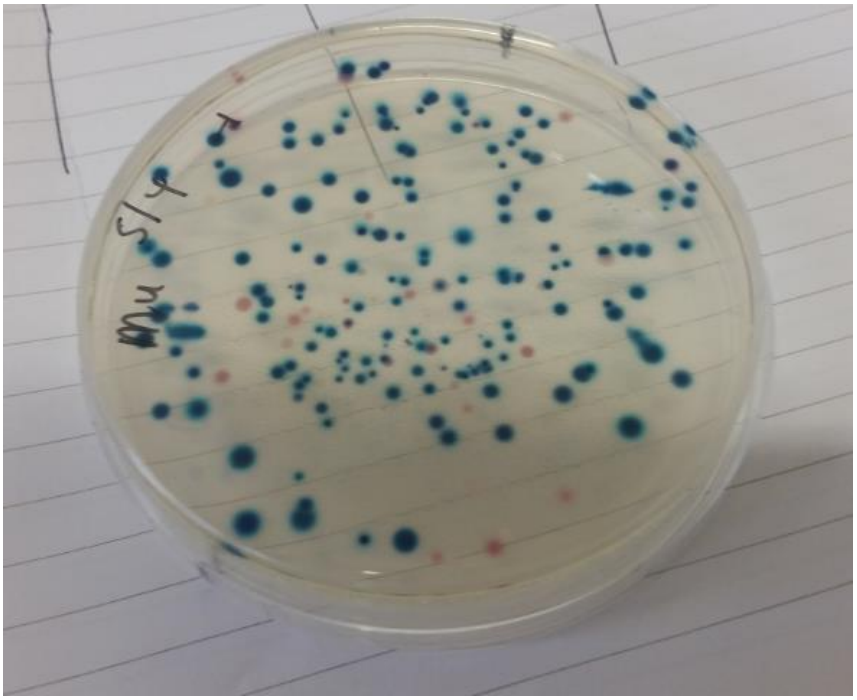


Figure 4-6 Growth on HiChrome agar showing bacteria colonies; Salmon red for faecal coliforms and purple for *E. coli*.

Plates with colonies within the range 30-300 were counted (APHA 2003) using the formula:

$$\text{count} \times 5 \times \text{dilution factor in colony forming units (cfu)}/ml$$

4.3.7.1.9 Identification

Identification of bacteria at final stages of treatment to observe FC either persistent through treatment regrown was done by use of prepackaged biokit Analytical profile index 20 Enterobacteracea (API 20E). In order to use the API 20E kit, pure cultures colonies of faecal coliforms and *E. coli* were prepared by sub culturing bacteria unto nutrient agar as indicated in section 4.3.4. Nutrient agar was prepared by adding 14 g of Nutrient agar powder into 500mL of distilled water and sterilizing in autoclave for 15 mins at 121°C. Agar allowed to coll to 55°C was poured into petri dishes and left to solidify. Colonies of faecal coliforms and *E. coli* from mixed culture on HiCrome agar were transferred to separate nutrient agar plates by streaking with sterile loops. Labelled plates were incubated at 37°C for 48 so that sufficient growth of bacteria would be possible.

There after a 0.5 Mcfarland standard used for estimating the concentration of bacteria in suspensions (Sutton 2006) was prepared by adding 9.95ml of 1% sulphuric acid to 0.05 ml of 1% barium chloride and votexing so that a cloudy

precipitate of barium sulphate was formed. The tube was placed on test rube holder. Accuracy of 0.5 Mcfarland standard colour could also have been checked by spectrophotometry. The absorption at wavelength 625 nm is between 0.8-1 with a 1cm light path of spectrophotometer (HealthLink 1999).

Test tube of same size as that used for 0.5 Mcfarland was used to prepare inoculum by suspending colonies from freshly prepared pure culture plates into 5ml Ringer's solution. This test tube was place besides the 0.5 Mcfarland standard and turbidity visually observed and adjusted till turbidity became identical to that of 0.5 Mcfarland standard. Principally the concentration of bacteria cells suspended in Ringer's solution medium partly absorbs and transmits light in all directions appearing milky in visible light so that biomass concentration could be estimated by comparing colour to a standard (Zamora and Gracia 2012). The density of bacteria in a suspension with the same visual turbidity as the 0.5 Mcfarland is said to be 1.5×10^{8} cfu/mL (Himedia ND) which is sufficient for use with API 20E kit.

Each API 20E kit contained 21 biochemical test which are listed in table 4.1 below;

Table 5: API20E biochemical test and active ingredients (Adapted from Biomerieux 2002)

BiochemicalTest (Abbreviation)	**Active ingredient**
cytochrome-Oxidase (OX)	tetramethyl-p-phenylenediamine dihydrochloride
O-Nitrophenyl-B-D-galactosidase (ONPG)	2-nitrophenyl-ßDgalactopyranoside
Arginine dihydrolase (ADH)	L-arginine
Lysine decarboxylase (LDC)	L-lysine
Ornithine decarboxylase (ODC)	L-ornithine
Citrate utilization (CIT)	trisodium citrate
Hydrogen Sulfide production (H_2S)	sodium thiosulfate
Urease production (URE)	urea
Tryptophanedeaminase production	L-tryptophane
Indole production (IND)	L-tryptophane
Acetoin production (VP)	sodium pyruvate

Gelatinase production (GEL)	Gelatin (bovine origin
Glucose fermentation (GLU)	D-glucose
Mannitol fermentation (MAN)	D-mannitol
Inositol fermentation (INO)	inositol
Sorbitol fermentation (SOR)	D-sorbitol
Rhamnose fermentation (RHA)	L-rhamnose
Sucrose fermentation (SAC)	D-sucrose
Melibiose fermentation (MEL)	D-melibiose
Amygdaline fermentation (AMY)	mygdalin
Arabinose fermentation (ARA)	L-arabinose

Oxidase test was performed first. On filter paper about 3 drops of oxidase reagent (tetramethyl-p-phenylenediamine dihydrochloride) was place and 2-3 drops of suspension was added, and observation was made after 10 seconds for purple coloration as a result of production of indophenols. This gave an oxidase positive indication which indicated the possible presence of *Pseudomonas* spp*., Vibrio cholerae*, *Neisseria* spp., *Campylobacter* spp., *Helicobacter* spp*./ Haemophilus* spp*., Aeromonas* spp., or *Alcaligens* (Tankeshwar 2012)

Each API tray was labelled and dated to identify specimen after which 5 mL of distilled water was spread on surface of tray so that a moist environment was available to avoid strip from drying off. The strip was then placed on tray and inoculated by transferring bacteria suspension with use of pipette into the strip carefully by filling both tube and capsule for all other test. The CIT, VP and GEL and just the tube for ADH, LDC, ODC, H_2S, URE were after overlaid with mineral oil to create anaerobic conditions. The lid was placed over tray and whole inoculation set was place in an incubator at 37°C for 18-24 hrs.

Incubator set was taken out of incubator after time elapsed and specific colony identified by assessing colour formations formed because of response of inoculum contents to specific test on strip. For the TDA, IND and VP test TDA reagents, James reagent and VP 1 and 2 reagents respectively had to be added for complete reaction which was indicated by colour. To all test reactions results were displayed

by colour changes on test strip (figure 4.6) and to these colour changes numeric values were assigned (as instructed by manufacturer) and this recorded on a result sheet (figure 4.7). As instructed by manufacturer the addition and then grouping or resultant gives a seven-digit number which identifies a specific bacteria specie. The identity of this bacteria was obtained by assessing the database in the API 20E manual and the importance of each isolate in wastewater treatment was discussed.

Figure 4-7 Example of API 20E strips after incubation

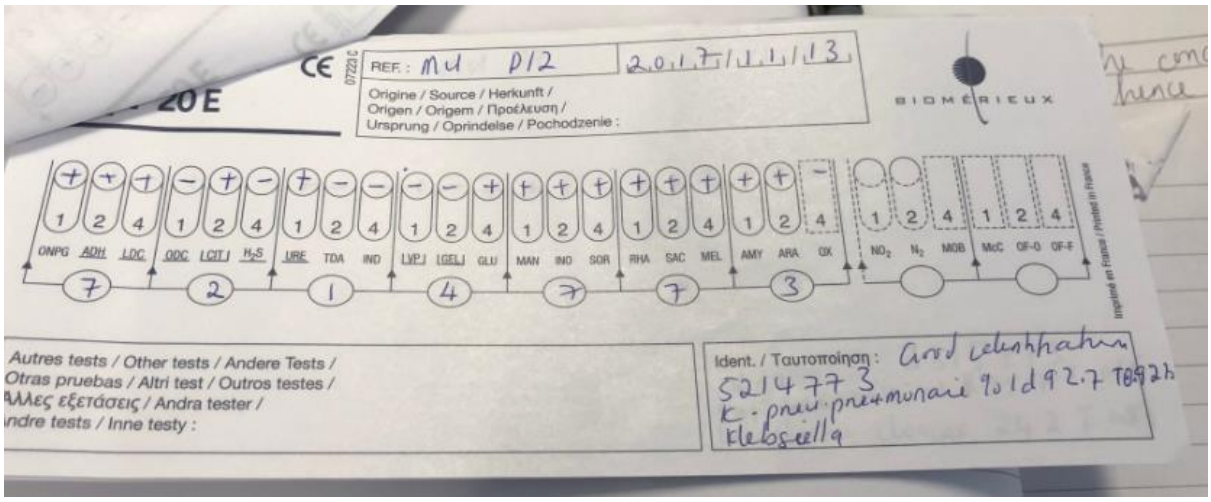


Figure 4-8 API 20E result sheet indicating how identification is obtained.

E. coli colonies were confirmed by reaction to drops of Kovac's reagent (Sigma Aldrich, UK) applied on pure colonies that were cultured on nutrient agar. In the presence of oxygen *E. coli* splits tryptophan into indole and α-amino propionic acid. Kovac's reagent contains hydrochloric acid and p-dimethylaminnocinnamaldehyde which combines with indole produced from the breakdown tryptophan to produce a red/cherry coloration (Bartram 1996)

Chapter 5. Carbonaceous Removal, Nitrogenous oxidation, and Pathogen Reduction in Nitrifying Activated sludge

5.1 Introduction

Domestic wastewater management is aligned towards reduction of carbonaceous substances, nutrients and pathogens and in biological treatment systems different microorganism are responsible for the degradation of these pollutants. In conventional activated sludge systems, the different processes take place in different reactors but in single sludge systems the microorganisms responsible for both processes are mixed in one reactor (Wang and Shammars 2009) and function together throughout the treatment time. The aerobic biological system's performance is subject to influence by physical factors of environment, chemical nature of water constituents such as dissolved oxygen and reaction substrate as well as system design, temperature, pH, constituents as discussed in section 2.2.4. These factors affect biodegradation activities of nitrifiers and heterotrophs responsible for nitrification and carbon oxidation. However, the variability in constituent of domestic wastewater pathogens hence biota as a result of health status and type of factories in the municipality and geographic location (Dumontet 2001) implies that every municipal wastewater stream will vary in microbial composition hence extent of biological treatment.

The objective of this initial chapter was to determine the conditions affecting the biodegradation of carbonaceous organic matter, nitrogenous oxidation and pathogen reduction in a bench scale reactor treating municipal wastewater. Pathogen reduction was assessed by evaluating changes in numbers of widely used indicators of faecal pollution (Ashbolt *et al.* 2001; Bahrim *et al.* 2012) *E. coli* and faecal coliforms. Also, an evaluation of the effect of these processes on the types of pathogens present at end of treatment will be done by identifying the faecal coliforms available at late treatment stages.

5.2 Methodology

Municipal wastewater obtained from Hatton wastewater treatment plant was continuously aerated in set up represented in figure 4.4. During the extended aeration period in batch reactor as elaborated in section 4.3 above, grab samples

collected at intervals from reactor were filtered by gravity as indicated in section 4.3.3. and filtrate analysed for physico-chemical characteristic as describes in section 4.3.7.1. Also, at each interval, 2 mL of filtrate was transferred into 1.5 mL Eppendorf tubes and kept in fridge (4°C) for four hours to be used as inoculum of bacteria culture for enumeration and identification of *E. coli* and faecal coliforms bacteria as described in section 4.3.7.2.

Preliminary trial experiments were conducted to establish the time at which nitrifying activity could be expected in the system. This was necessary to establish the appropriate solid retention time for this system as mixed liquor source was non-nitrifying. With respect to this, aeration was carried out for 4 hours, 3 days and 5 days periods. One sub sample were therefore analysed every hour in 4 hour and every day during 3- and 5-day periods respectively. Analysis were carried out for physico-chemical parameters NH_4^+-N, NO_3^--N, NO_2^--N, pH, temperature and DO only during trial experiments. As trial experiments were conducted in triplicate each period three biological replicates of wastewater were therefore analysed each period.

Thereafter, seven runs of main experiment were carried out at established solid retention time. With a new sample from treatment plant used for each run, grab sampling from reactor, filtration, physico-chemical analysis and enumeration were carried out at intervals on day 0, 2, 4, 7, 9, 11 each. However, identification of bacteria was only done from bacteria colonies cultured with samples of day 9 and 11. Colonies of both FC and *E. coli* were obtained from mixed culture of colonies grown on HiChrome agar (Sigma Aldrich, UK) and used for culture of pure colonies on nutrient agar. Different species of FC were identified by use of API 20E (Biomerieux, UK) as elaborated in section 4.3.7.2.2. However, individual colonies of *E. coli* were confirmed by the addition of a drop on Kovac's reagent (Biomerieux, UK) on the surface of individual colonies.

Data was analysed with the help of SPSS version 23 (IBM 2015) statistical program. Spearman's non-parametric correlation coefficient test was used to establish correlation coefficient between variables relating to nitrification and pathogen reduction processes.

5.3 Results and discussion

Results of seven biological replicates of wastewater analysed are shown individually in figure 5:1- 4 and figure 5.5 represent average changes in wastewater constituent due to changing processes in reactor. Nitrate nitrogen increase in wastewater samples, indicating presence of nitrification, was not observed after the four hours or three-day treatment period but observations of nitrate increase were evident after the 5-day period of continuous aeration in single batch reactor. Therefore, hydraulic retention time was considered after 5-day retention period. However, this increase was stalled after 11days in all runs. Therefore, the solid retention time for main experiment was adopted at 11 days. This is within the stipulated period observed in previous research (Tang and Chen 2015; Von Spieling 2007; Burton *et al* 2014). Seven runs of experiment were carried out and seven biological replicates of wastewater from Hatton wastewater treatment plant were analysed for changes in carbonaceous removal, nitrogenous oxidation and FC numbers. These changes were derived from the concentrations of BOD_5, NH_4^+-N and the nitrogenous oxides as well as changes in faecal coliforms numbers observed (Figure 5.1, 5-2. 5-3 and 5-4 showing reactors A & B, B & C, E & F and G respectively) at interval mentions in 5.2 above during runs of the treatment, as nitrogenous oxidation proceeded. All runs showed similar trends of reduction in BOD_5, initial increase then reduction in NH_4^+-N and gradual increase in NO_3^--N which is identical to that expected as represented in figure 4.2. The overall change in wastewater character was represented in table 5.2 and figure 5.2 i & ii.

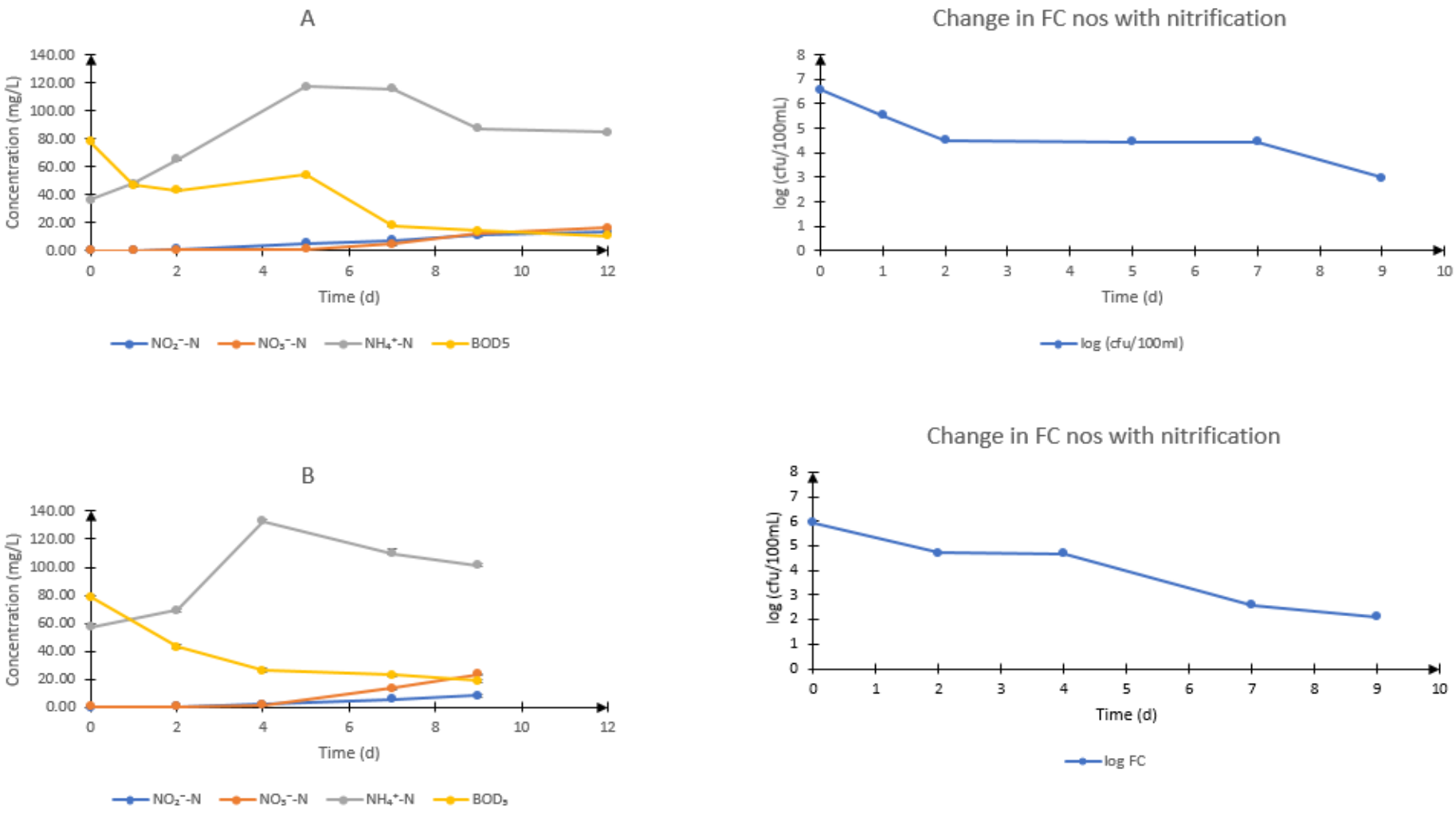


Figure 5-1 Average change in wastewater character alongside change in FC numbers in runs A and B (Error bars indicate standard deviation)

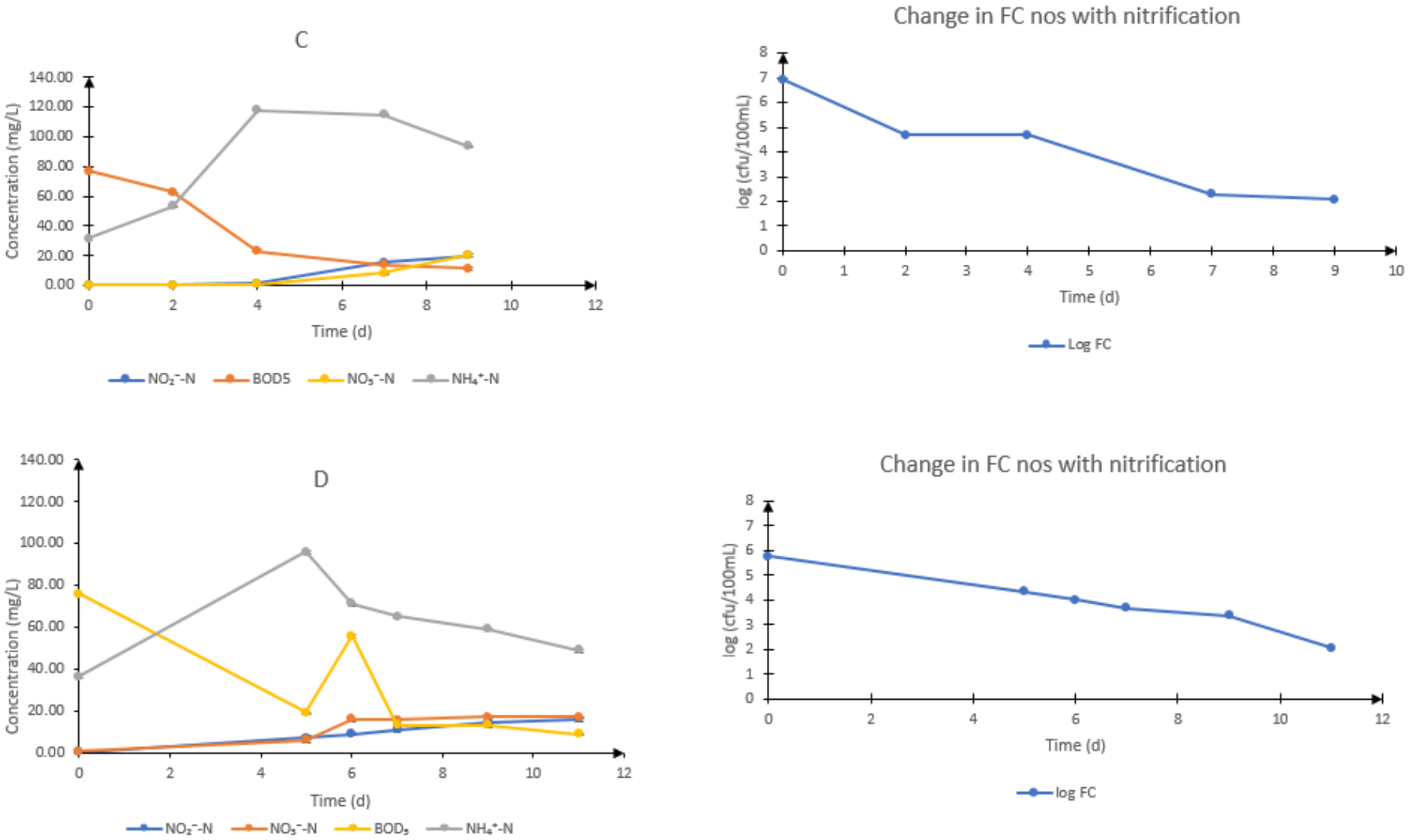


Figure 5-2 Average Change in wastewater character alongside change in FC numbers in runs C and D (Error bars indicate standard deviation)

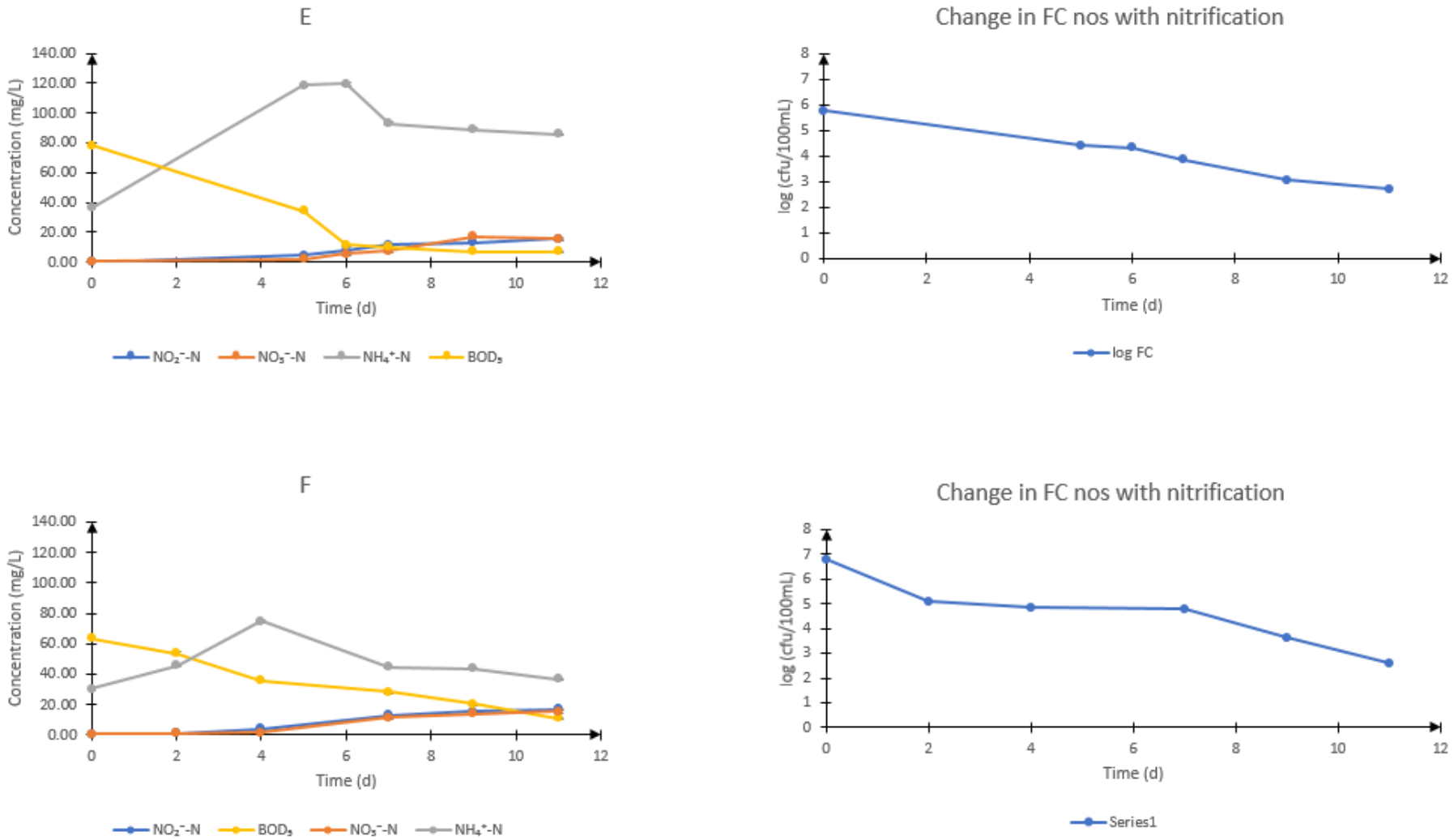


Figure 5-3 Average change in wastewater character alongside change in FC numbers in runs E and F (Error bars indicate standard deviation)

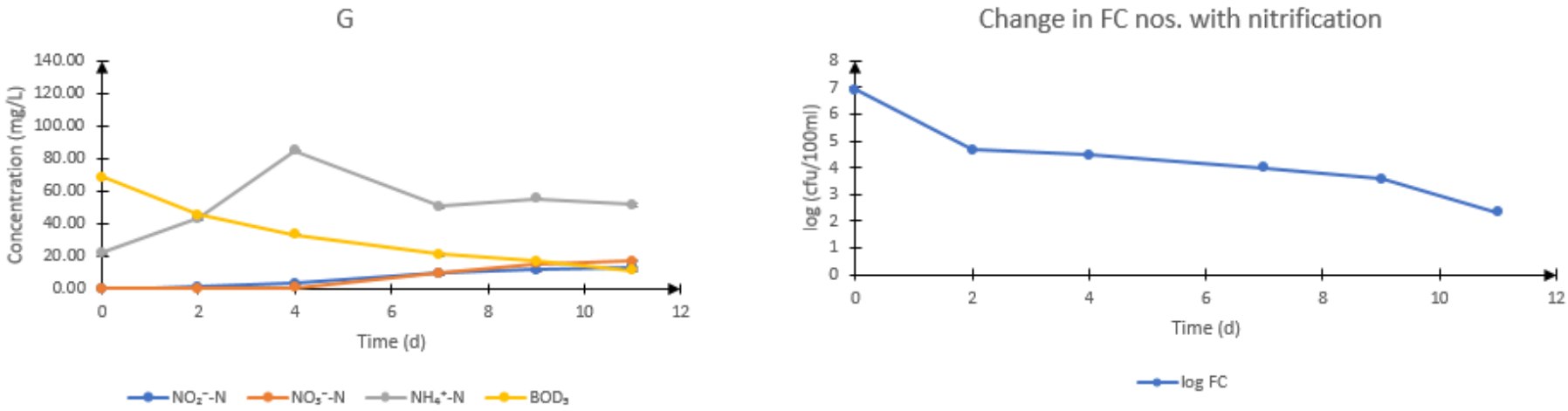


Figure 5-4 Average change in wastewater character alongside change in FC numbers in run G (Error bars indicate standard deviation from mean)

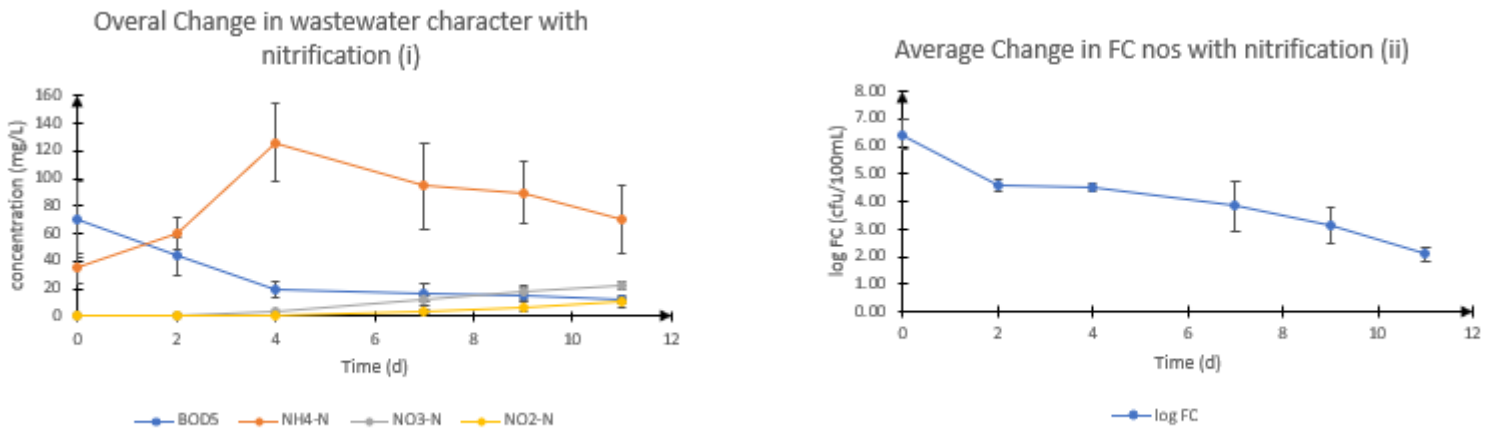


Figure 5-5 Average change in wastewater character and faecal coliform (FC) after treatment.(Error bars indicate standard deviation from meam, n=7)

Table 6 Change in wastewater characteristics through treatment

Time (days)	T (ºC)	pH	DO (mg/L)	BOD_5 (mg/L)	NH_4^+-N (mg/L)	NO_3^--N (mg/L)	NO_2^--N (mg/L)	log FC
0	19.2±1.7	7.4±0.2	1.0±1.3	69.9±27.8	35.3±10.9	0.4±0.1	0.03±0.02	6.4±0.5
2	22.1±1.1	8.0±0.8	5.63±1.1	43.6±13.4	59.7±11.8	0.6±0.2	0.1±0.4	4.6±0.2
4	22.8±0.1	7.8±0.4	5.3±1.4	19.3±6.2	106±27.8	3.2±0.5	1.3±0.3	4.5±0.2
7	21.0±0.4	7.3±1.2	6.8±0.2	17.2±6.3	94.3±31.2	11.9±3.6	3.4±1.1	3.9±0.9
9	22.6±0.7	6.2±1.1	7.9±0.7	15.4±4.9	89.7±22.5	17.5±4.7	6.9±1.8	3.1±0.7
11	20.6±1.6	6.5±0.4	8.9±0.8	13.1±2.2	69.8±24.6	22.4±3.2	10.3±2.1	2.1±0.3

Average temperatures and pH were 21.53°C and 7.21 respectively which were within the ranges observed for effective nitrification activity in previous works (Cui *et al.* 2014; Coskuner and Jassim 2008; Huang *et al.* 2010; Tang and Chen 2014; Kumari *et al.* 2011). Continuous aeration and mixing resulted in dissolved oxygen concentration within the range of 0.97 to 8.87 mg/L averagely through the length of treatment rising above the optimal ranges for both carbonaceous matter reduction and nitrification in the batch reactor. As observed therefore, these physical factors temperature, pH and DO were within standard values for carbonaceous pollutant reduction and nitrogenous pollutant oxidation implying that they pose no hinderances to processes so that other factors are responsible for changes observed.

5.3.1 Carbonaceous matter removal

A general reduction in BOD_5 was observed implying that the oxygen demand for oxidation of carbonaceous matter reduced as treatment progressed (Table 5-1 and figure 5-5i). This indicated that the quantity of biodegradable organic matter in wastewater reduced through treatment time. Precisely there is an initially sharp decrease from 69.89 mg/L to 19.34 mg/L averagely in BOD_5 within the first 5 days and thereafter much slower reduction from 19.34 to 13.09 mg/L is observed (Table 5.1). Probably heterotrophs in added mixed liquor reproduce rapidly as they consume available organic matter and because flow is not continuous organic matter concentration reduces within this time (0-4 d). This activity is therefore shown in a resulting decrease in biochemical oxygen demand measured as BOD_5. Parallel to this activity a corresponding decrease in average FC numbers is observed (figure 5.5ii) implying that at this stage faecal coliform reduction was possibly caused by a reduction in the availability of organic carbon.

5.3.2 Nitrogenous oxidation

Changes in the concentration of substrate as NH_4^+-N and products NO_3^--N and NO_2^--N in the system were the variables used to assess nitrogenous oxidation as observed in previous studies (Coskuner and Jassim 2009; Kumari *et al.* 2011; Tang and Chen 2014). Previous research (Princic *et al.* 1998) suggest two prominent factors affecting the activity of nitrifiers are substrate (NH_4^+-N) concentration and organic carbon concentration. An average increase in NH_4^+-N from 35.33 to106 mg/L after 5 days of treatment indicated that ammonium was being added to the system. This increase indicated that at this stage ammonification of organic nitrogen was still

ongoing. Despite the availability of sufficient supply of oxygen, NH_4^+-N appeared not to be utilised. Coincidentally relatively low quantities of nitrogenous oxides 0.4 to 0.5 mg/L (figure 5.5 and table 5.1) were also observed indicating possibly that nitrogenous oxidation had not commenced at this stage (before day 4) hence another reason for high concentrations of NH_4^+-N. Competitive advantage for oxygen by heterotrophs caused oxidation of carbonaceous matter, indicated by reduction BOD_5 values, to override oxidation of nitrogenous matter at this stage. Therefore, though ammonium is substrate, nitrification was more influence by carbonaceous reduction as a result heterotrophic activity than substrate concentration before day 4 of treatment. Also, nitrifiers are slow-growers' sensitive to environmental changes (Li *et al.* 2014) so it is possible that the few found in mix liquor would have been adapting to the new environment in batch reactor thereby reducing chances for the occurrence of nitrification at this stage.

However, after this period (day 4) a continuous decrease in NH_4^+-N concentrations and corresponding increase in NO_3^--N values were observed thereby indicating that nitrogenous oxidation was ongoing (Figure 5.5ii). The occurrence of these activities and the steadily low concentrations of BOD_5 infer that at this stage of treatment, carbonaceous matter had been sufficiently reduced by heterotrophs, reducing competition for oxygen thereby allowing for growth of nitrifies hence nitrogenous oxidation. Also, pH reducing from 8.04 to 6.22 from day 5 indicated gradual loss of alkalinity in the system and confirm nitrification was taking place. This reduction in pH confirms previous research which suggested that a drop in pH was inevitable in nitrifying systems in which no liquid replacement was done as in batch reactors (Pogue and Gilbride 2007)

On assessing the relationship between changes NH_4^+-N and NO_3^--N concentration through the treatment system, Pearson's correlation (SPSS version 23), indicates no linear correlation existing ($r=0.191$, $p= 0.717>0.05$). Possibly because of the increase before decrease in NH_4^+-N in the system or could also reflect the differences shown by different nitrifying bacteria to quantities of ammonium as observed in previous research (Almstrand *et al.* 2011). This implies that the concentration NH_4^+-N despite being substrate of nitrification doesn't indicate the occurrence of nitrification throughout the system nor affects reduction of FC in the treatment system.

However, Pearson's correlation (IBM 2015) indicated a significantly negative correlation (-0.907, p=0.013<0.05) between the concentration of NO_3^--N and log FC (Table 5.2) through treatment. Also, on assessing the effects of nitrogenous oxidation on FC concentrations (figure 5.5i &ii) after day 4 it is observed that an increased in NO_3^--N concentration resulted in a corresponding decrease in log FC. These all indicate that the presence of NO_3^--N appears to have an impact on the concentration of FC numbers.

Table 7: Correlation between wastewater characteristics relevant for nitrification and pathogen reduction

Correlations

		BOD_5	NH_4^+-N	NO_3^--N	Log FC
BOD_5	Pearson Correlation	1	-.754	-.749	.884*
	Sig. (2-tailed)		.083	.086	.019
	N	6	6	6	6
NH_4^--N	Pearson Correlation	-.754	1	.191	-.375
	Sig. (2-tailed)	.083		.717	.464
	N	6	6	6	6
NO_3^--N	Pearson Correlation	-.749	.191	1	-.907*
	Sig. (2-tailed)	.086	.717		.013
	N	6	6	6	6
Log FC	Pearson Correlation	.884*	-.375	-.907*	1
	Sig. (2-tailed)	.019	.464	.013	
	N	6	6	6	6

*. Correlation is significant at the 0.05 level (2-tailed).

Nitrite was also seen present in the system as evidence of nitrogenous oxidation. The accumulation of nitrite is not supposed to occur in nitrifying systems as nitrite is substrate for nitrate production and therefore immediately used up (Schramm *et al.* 2000) however nitrite was observed in this system. Previous scientist (Keller *et al.* 2002) who worked with batch reactors had observed that nitrite build-up in their systems were as a result of the presence of high quantities of ammonium. High quantities of ammonium in this system could therefore be responsible for the accumulation of nitrite observed.

Mixing in the system by activity of the magnetic stirrer and stirrer bar as well as agitations as a result of aeration activities ensured that ammonium and oxygen were

uniformly distributed in system so that nitrifiers were not starved of ammonium or oxygen at any point in the reactor in order to achieve optimal oxidation of NH_4^+-N. However, NH_4^+-N levels though reducing were still high (69.75 mg/L) by end of treatment which implied that oxidation was not optimal. This could be an indication of the slow growth of nitrifying bacteria before end of treatment despite the presence of favourable conditions of low organic carbon, ammonium nitrogen and oxygen hence an indication of the presence of insufficient amounts of nitrifiers or the presence of partial inhibition.

Previous research indicates that the two types of nitrifiers have different growth rates with AOB growing faster at lower oxygen concentrations than NOB (Peng and Zhu 2006) and respond to ammonia nitrogen concentrations differently (Almstrand *et al.* 2011). Also, AOB which are starters of the process are more sensitive to environmental change than NOB (Bellucci *et al.* 2011). The use of mixed liquor from full-scale Hatton wastewater treatment plant to laboratory batch reactor meant that the environmental conditions to which the bacteria were exposed where changed so that though environmental conditions of pH, temperature and DO were favourable, adaptation of organism to the new environment might have affected the AOB hence the optimisation of the process. More so, both type of nitrifiers are said to exhibit differing affinities to different concentrations of NH_4^+-N so that NOB require three times more NH_4^+-N than AOB (Princic *et al.* 1998) implying that enough ammonium must be available for both steps to be optimised. However, the presence of nitrite in system (table 5.1) indicated that ammonia oxidation was ongoing, but nitrite oxidation appeared to be slow. This could be an indication of the formation of sludge floc whose presence is said to result in peaks of nitrite concentration due to slow growth of NOB (Philips *et al.* 2002). Also, previous studies indicated that the presence of high concentrations of ammonia result in limitation of nitrification due to the presence of free ammonia (Anthonisien *et al.* 1976) which has also been identified as responsible for presence of nitrite in close culture systems (Volslarova *et al.* 2008). Despite the effects of nitrite and free ammonia in previous studies their effect on FC numbers in this system is not clearly understood and would require further investigations.

5.3.3 Pathogen Reduction

The filtering of wastewater (section 4.3.6) retains sludge formed by microorganisms and suspended particles involved in the treatment of dissolved substances (Malham *et al.* 2013; Baweic *et al*. 2016) so that filtrate samples used for analysis would account for just those FC persisting in the water phase. Pathogen reduction is seen in reduction in number of colonies of faecal coliforms counted on HiChrome agar (Table 5.1 and Figure 5.5ii). From 6 log to 2 log therefore implying that during both processes of carbonaceous removal and nitrogenous oxidation there was reduction in pathogen numbers. This confirms Bentecourt and Rose (2006) who state that as wastewater treatment proceeds gradual reduction of pathogens occurs. From day 0-4 pathogen reduction appeared to be influenced by BOD_5 removal processes while removal from day 4 onwards was influence by processes involved in nitrogenous oxidation (Figure 5.3).

This reduction of FC numbers observed before day 5 occurs as a result of the reduction in the municipal wastewater's rich organic carbon content (Anderson *et al*. 2005) as wastewater retention time increases in the batch system. Die-off of microorganisms because of starvation is said to occur (Webbe and Legge 2008; Kadlec and Knight 1996) as quantities of resources which support growth decrease. This is further explained in Figure 4.1A which illustrates the possible changes that occur as wastewater with organic carbon is detained over time in aerated conditions. Microorganism hereby are seen to aggregate and form floc seemingly in response to reduction in organic carbon contents. More so, a reduction in quantities and type of pathogenic organisms like *E. coli* and enteric bacteria has been observed in aquatic environments with less quantities organic matter (Wanjuyi and Harwood 2013) as observed in the batch system hereby. Natural die-off could be possibly responsible here as decrease in organic carbon (food) measured as BOD_5 would result in starvation and occurs parallel to reduction in faecal coliform numbers (Figure 5.6).

After day 4 (indicate by point A in fig 5.6), pathogen in wastewater are expose to a relative higher concentration of nitrogenous oxides; nitrates and nitrites. Nitrites which are substrate for nitrate formation have been observed to be antimicrobial (Carrington 2001). Their presence in wastewater systems has been observed to be toxic disrupting biological processes (e.g. ammonia and nitrite oxidation) as they are said to intervene with enzyme functions (Philips *et al.* 2002). As nitrate and nitrite

concentrations increase in the present system, concurrent and further reduction in FC numbers were observed (Figure 5.5). Though FC numbers reduce further at this stage, it is not very clear if it is the presence of the oxides that is responsible for this hence further investigation will be carried out in chapter 6. Using one way Anova (SPSS, IBM 2015) to compare average reduction in FC at day 2 and day 4, at $F(1,12)$, $p=0.86$ at $\alpha=0.05$ indicating no statistical significant difference between average reduction at day 2 and average reduction at 4. Reduction therefore about these points appeared to have levelled off. This implies therefore that reduction before and after day 4 appeared to have resulted from different causes.

Much more, Carrington (2001) states that the activities of other wastewater micro flora like predatory bacteria and protozoa have been observed to exert a major impact on the reduction of pathogens at ambient temperatures in AS systems. Protozoa particularly are known as major predators in WWTS whose activities in aquatic systems has led them to be called disinfecting agents (Papadimitriou *et al.* 2010). It is therefore possible that their presence could have contributed in the reduction of FC in this system. Further investigation will be carried out in chapter 7 to ascertain what impact they might have on nitrification in this system as well as FC reduction.

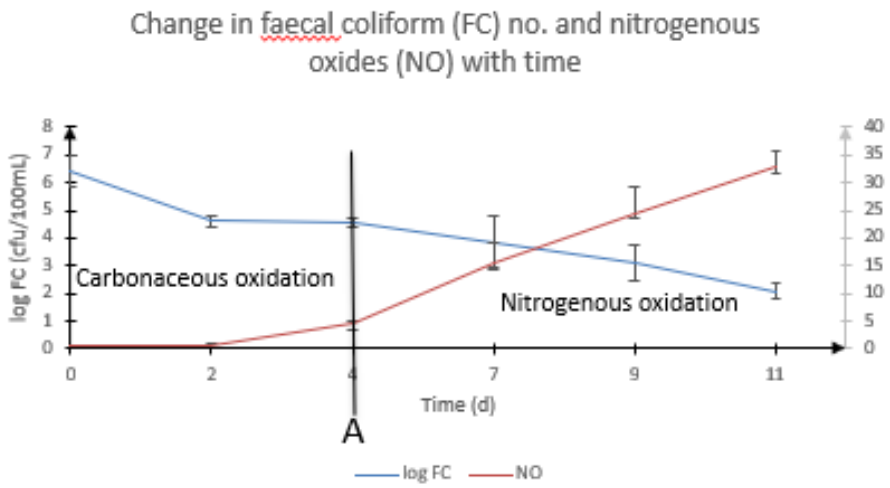


Figure 5-6: Faecal coliform removal stages with time (Error bars indicate standard deviation from mean, NO is total nitrogenous oxides)

5.3.4 Identification

With the use of the Analytical Profile Index (API 20E) system, the identified Gram-negative organisms observed in samples of days 9 and 11 are represented in the figure 5.5 below. Of the 50 strips used, 35 profile numbers were obtained, 27 corresponded to an identity on the API 20E manual and 8 did not correspond. Though Gram-negative and already identified by colour on HiCrome agar, *E. coli* was not identified by API 20E as the profile number obtained could not be matched in the manual. This confirms the observation by O'hara *et al.* (1999) who suggested that situations as this were evidence of lack of accuracy in the API 20E system observed more at the 24hrs incubation period than at the 48hrs period. However, all strips allowed for 48hrs incubation period dried up and could not be identified hence suggestion above could not be tested in this instance. Instead, *E. coil* presence was confirmed by pink coloration on pure cultured colonies after 2 drops of Kovac's reagent was applied on them. This pink coloration was observed on all pure cultures of *E. coli* tested.

Of the bacteria species whose presence is important in municipal WWT effluent (Section 3.2.1), *Pseudomonas aeruginosa*, *P. fluorescens*, *Enterobacte*r *cloacae, Salmonella* spp. and *E. coli* were amongst those identified in the present system (figure 5.7). Though others were not stated as important, their presence are indicative of processes through which wastewater has undergone as well as indicative of faecal contamination by other organisms hence are considered as pathogens e.g. *Klebsiella* spp., *Citrobacter* spp. and *Chromobacterium* spp. (Ashbolt 2001). The significance of the presence of some important as water pathogens are evaluated hereby.

Pseudomonas aeruginosa is an obligate aerobe which can grow between 25°C-37°C but is distinct from the other *Pseudomonas* spp. as it also grows at 42°C (Wu *et al.* 2015) indicating its persistent nature. It is an important pathogen to plants and animals causing cystic fibrosis in immune-compromised humans. *Pseudomonas* spp. were identified as facultative anaerobes in WW treatment (Khan *et al.* 2013) but *P. aeruginosa* is said to grow at lower DO levels than either *P. fluorescens or P. putida* whose presence indicated oxygen availability. Some authors suggest *Pseudomonas aeruginosa's* presence is indicative of aerobic denitrification (Chen *et al.* 2003) whereas others identify it to contributing to heterotrophic nitrification in AS systems

(Cydzik-Kwiatkowska *et al.* 2015). Its diversified involvement in wastewater processes possibly explains its persistent nature through treatment processes.

The presence of *Klebsiella oxytoca* and *K. pneumonia* which have also been identified in soil, uncontaminated fresh and saltwaters (Cabral and Marques 2005) confirm on going nitrification. *K. oxytoca* is a nitrite oxidising bacterium (Abd-al-haleem *et al.* 2007) while *K. pneumonia* has been isolated in overly aerated wastewaters (Bowers *et al* 2008). As pathogens they're responsible for incidence of pneumonia and urinary tract infections in elderly.

Enterobacter sakazaki responsible for sepsis and meningitis in infants is said to be involved in the degradation of organic matter as well as aggregation of bacteria due to reduced organic carbon as it indicates that a stabilizing community is being formed (Turki *et al.* 2016).

Salmonella spp. are pathogens linked to gastrointestinal illnesses and dysentery in humans (WHO 2011). Earlier research suggest that *Salmonella* is usually reduced to undetectable level in secondary effluent (Mostoe *et al.* 2013) which is contrary to what is observed here. They were the most detected FC in this system and other authors state that AS systems are unable to completely remove them (Boulani *et al.* 2017). This might be a possible reason for their quantities here.

As observe the quantities of FC were affected by both processes of carbonaceous matter reduction and nitrogenous oxidation in the AS system confirming the results of Princic *et al.* (1998) which indicate that communities of microorganisms are affected by changes in concentrations of oxygen, pH and ammonium, all of which occurred in this system. Noticeable is the dual importance of some of the faecal coliforms as participating in organic carbon degradation e.g. *Enterobacter sakazaki* and nitrogenous oxidation e.g. *Klebsiella* spp. Therefore, progress of these processes will affect and reduce the numbers of these bacteria available as substrate reduce in batch system hence reducing the FC numbers in the system. However, identification of FC in early stages of experiment for comparison with those identified in latter stages is recommended to provide a better picture on the effect of carbonaceous reduction and nitrogenous oxidation on FC herein.

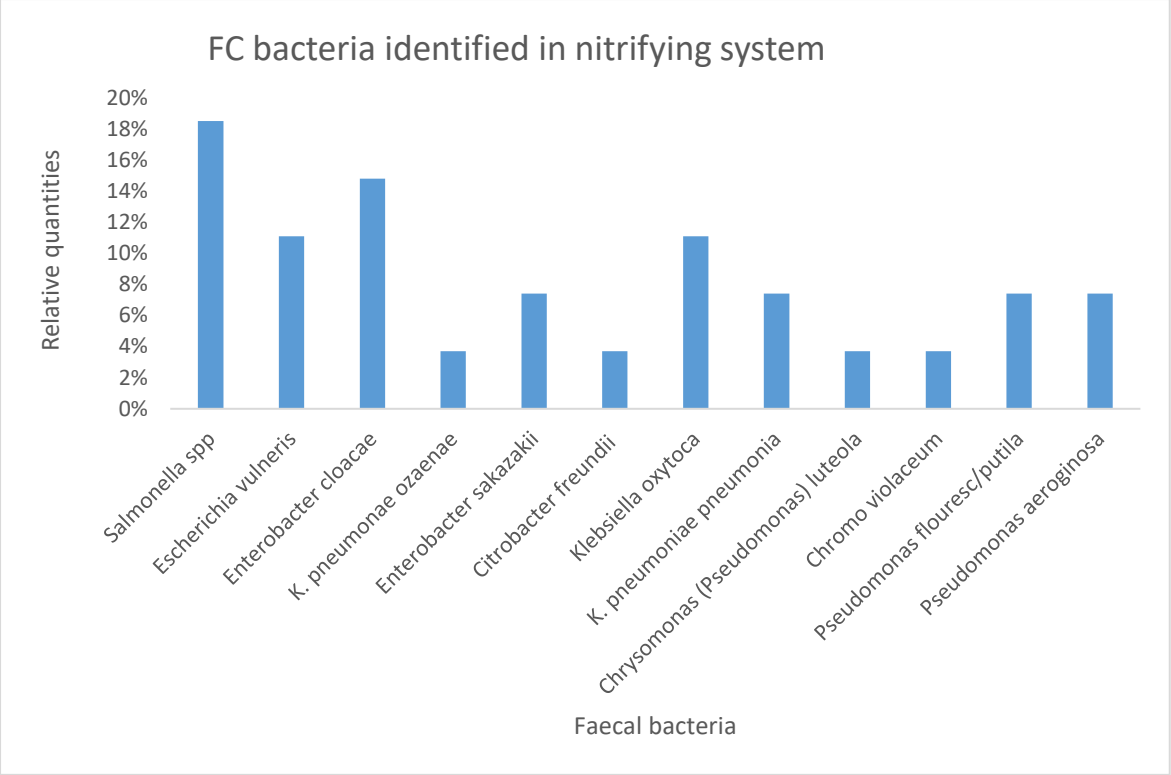


Figure 5-7 Bacteria identified at final stages of batch treatment by API 20E

5.4 Key points and Conclusion

Changes in faecal coliform quantities were observed in this system as the processes of carbonaceous reduction and nitrification took place one after the other. Initially the reduction could be attributed to reduction of organic carbon but at later stages reduction was attributed to nitrogenous oxidation. At average ambient temperature of 21°C and dissolved oxygen constantly above 2 mg/L a solid retention time of about 11days was necessary for nitrification to level off. Nitrification was preceded by the process of organic carbon reduction and during this time there was accumulation of ammonium nitrogen. Inorganic nitrogen oxidation was confirmed by decrease in pH, increase in the concentration of nitrite nitrogen and nitrate nitrogen.

FC reduction occurred concurrently with both processes and was initially attributed to reduction in organic carbon concentration leading to die off. However, when the reduction of organic matter levelled off, further reduction of FC was attributed to the processes of nitrogenous oxidation. The process of carbonaceous matter reduction in the AS also resulted to the formation of sludge to which some microorganisms may have been attached and subsequently removed from the system by filtration.

This experiment has established the fact that the factors responsible for nitrification in laboratory scale AS batch reactors also lead to a reduction in FC number. Possibly giving a lead to the hypothesis that nitrification and pathogen reduction occur concurrently. However, though reduction in organic carbon results in FC reduction as a result of lack of food or adsorption on formed microbial flocs the reason for further reduction observed is not clearly understood.

Clearly organic carbon concentration levelled off and because samples were filtered any reduction at these latter stages could be attributed to nitrification or the impact of any other constituent of the wastewater community. Further investigation was therefore essential to establish the possible effects of the products of nitrification as well as the possible effect that the major predators, protozoa, may have on FC numbers in this system. These investigations will be considered in Chapter 6 and chapter 7 respectively.

Also, the API 20E system of identification revealed the presence of *E. coli* and several faecal bacteria at latter stages of nitrification. An observation of the dual character of several FC as pathogens and involved in either organic carbon reduction or nitrogenous oxidation was made. Hence the progress of these processes should definitely result in the reduction in quantities of these organisms in the system as substrates decrease.

Chapter 6. The Effects of Nitrogenous Oxides on Faecal Coliforms Quantities in Activated Sludge

6.1 Introduction:

The occurrence of nitrogenous oxides at secondary treatment is prominent after the reduction of organic carbon as observed in section 5.3. In this aerobic wastewater treatment system, the reduction of organic matter content of municipal wastewater resulted in reduced competition for oxygen, allowing for the oxidation of inorganic nitrogen. Time lapse also gave the slow growing and sensitive nitrifiers present in the mixed liquor an opportunity to grow and stabilize so that when competition for oxygen was reduced, oxidation of ammonia which should result in the presence of nitrite and nitrate occurred as observed after day 4 in chapter 5.

Biological nitrogen removal processes are said to be the main sources of nitrite and nitrate in the aquatic environment (Smith *et al*. 1997). Nitrogen species therefore present at secondary treatment are NH_4^+-N, NO_2^--N and NO_3^--N which are transient as they are all substrates for the respective next stages of nitrogen removal. All three of these have been identified as directly toxic as elevated quantities have been seen to inhibit biological processes (Carmago and Alonso 2006). In nitrification, nitritation is the rate limiting step (Wett and Ruach 2001) and nitrite accumulation has been related to many factors (section 2.4.1) including excessive ammonia loading of system, limited supply of oxygen and low temperature (Philips and Verstraete 2000). The presence of free ammonia (FA) due to the dissociation of NH_4^+ to NH_3 as a result of increase pH has been observed to cause the accumulation of nitrite as FA inhibit nitrite oxidation (competitively inhibiting nitrite oxidoreductase enzyme) thereby causing the accumulation of nitrite in some WWTS (Yang and Alleman 1992). However, nitrifiers have been observed to get acclimated to FA so that nitrite build-up is transient (Turk and Marvinick 1989). The occurrence of nitrification reduces pH of the system as concentrations of NH_4^+-N reduce due to oxidation and H^+ are release with the formation of NO_2^- as expressed in equation 2.4. NO_2^- is said to exist in equilibrium with free nitrous acid (FNA) and as pH reduces further, FNA concentration increase (Anthonisen *et al.* 1976). Nitrite oxidation is inhibited by FA at pH >7 while at pH < 7 FNA is responsible for nitrite oxidation (Philips *et al.* 2002). The action of FNA on nitrite oxidation has been observed to be as a result of its

inhibition ATP synthesis thereby inhibiting cell metabolic processes (Glass *et al.* 1997).

Also, considering that the rate of consumption of nitrite is usually twice the rate of production, there should be no accumulation of nitrite. Rather, growth rates and different sensitivities of AOB and NOB to environmental changes and substrate implies this does not usually occur. Instead, as nitrification progresses peaks of nitrite result as a result of slower growth of NOB to AOB initially (Phillips *et al.* 2002). More so, the NOB are more sensitive to environmental change than AOB (Muirhead and Appleton 2008) so that their inhibition results in build-up of nitrite.

Nitrite has been known to be a bacteriostatic molecule associating with metals in enzymes thereby hampering enzyme-controlled reaction leading to inhibition of growth and reproduction of bacteria (Wild *et al.* 1995). In wastewater treatment systems its presence has also been associated with the inhibition of biological reactions due to its ability to increase the proton permeability of cell wall so that ATP synthesis, hydrolysis and reactions catalysed by ATPase decrease. Increased toxicity was observed when nitrite was added to AS containing amino acids and proteins (Philips and Verstrate 2000).

However, its presence has been useful in other wastewater treatment technologies such as the SHARON (Single reactor system for High activity Ammonium Removal Over Nitrite) and the ANAMMOX (Anaerobic Ammonia Oxidation) (Phillips and Verstate 2000). More so nitrite addition to sludge has been shown to be beneficial to pathogen removal (Du *et al.* 2017). Previous investigations with addition of nitrite to AS indicated increase in toxicity when amino acids or proteins where added along (Philips and Verstrate (2000). The present study investigates the potential benefits of its presence during nitrification to the reduction of pathogens.

This chapter deives its aim from figure 5.6 and sets to investigate the effect of nitrogenous oxidation by nitrification may have on the FC numbers in a batch aerobic activated sludge treating municipal wastewater.

6.2 Methodology

In order to evaluate the effect of nitrogenous oxidation on faecal coliforms in the system, four continuously and extensively aerated batch reactors were used in

parallel. The setup was same as described in section 4.2 and municipal wastewater working volume in both reactors was 3 L. Treatment in all reactors was basically the same as in section 5.2 but an additional modification was made in three reactors. The experimental design was elaborated in table 6.1.

Wastewater was allowed to aerate till DO saturation point so that sufficient oxygen was available for aerobic activity before addition of chemicals. Reactor A was the basic extended batch aeration system established in section 5.3 used as reference. No chemicals were added into it.

In reactor B 3.33 ml/L allylthiourea (ATU), a nitrifying inhibitor, was added (Progue and Gilbridge 2007) at the onset of aeration to inhibit any nitrogenous oxidation. Addition of ATU was important to ensure that response of biota to added nitrite or nitrate will not be camouflaged by any on-going nitrifying activity. As a selective inhibitor (ATU) of nitrifiers metabolic process it ensured that no ongoing production of nitrite and nitrate so that any increase in nitrite or nitrate will be as a result of the added chemicals.Also other organisms in the mixed population of wastewater bacteria could carry on their normal functions (Ginestet *et al.* 1998). ATU inhibits ammonia oxidation selectively by coupling with the copper of the ammonium monooxygenase enzyme's active site hence preventing the process of ammonia oxidation. This inhibition is spontaneous and complete to ammonia oxidisers at concentrations between1-10 g/L (Surmacz *et al.* 1996) and allylthiourea is not known to affect other biological processes in the system (Bedard and Knowles 1989). However, when it is used at concentrations above 2 mg/L increase in BOD_5 measurements have been observed (APHA 1989). Inhibition of nitrification in AS could also be done by inhibiting nitrite oxidation with chlorate but its action is said to be slow and non-specific (Hynes and Knowles 1983). Also added to B were sodium nitrite (Merck UK) a common additive in the food processing industry and sodium nitrate (Merck UK) used as a nitrogen source for aquaculture ponds and is highly soluble in water (Boyd 1997). In properly controlled nitrification processes nitrite does not exceed 1 mg/L and at this level it is said to be toxic to aquatic organisms (Muirhead and Appleton 2008) hence 4.9 mg/L of sodium nitrite was added to mixture to give 1 mg/L NO_2-N. Concurrently 60 mg/L of sodium nitrate was also added to the same mixture so as to provide 10mg/L of NO_3-N. The latter is the safe maximum concentration of nitrate in surface waters (Team P.S.A 2005) and

therefore a reasonable amount in treatment system. In this study the addition of sodium nitrite and nitrate together was to achieve the presence of the oxide NO_3^- and NO_2^- via oxidation in the same system as it would be in a nitrifying system as observed in fig 5.3. In reactor C just allylthiourea was added to the sample to assess change in FC concentration without the presence of increased concentration of nitrogenous oxide. Finally, in reactor D the sodium nitrite and sodium nitrate were added without any addition of allylthiourea so that the effect of added oxides without the presence of the inhibitors was assessed.

Table 8: Experimental methodology

Reactor	***A (n=6)***	***B(n=6)***	***C(n=6)***	***D(n=6)***
Extended aeration	Yes	Yes	Yes	yes
Allylthiourea	No	Yes	Yes	No
Addition of $NaNO_3$ + $NaNO_2$ after 24hrs	No	Yes	No	yes

After day two, reactor A went through the whole treatment to ensure that sample collected was able to nitrify and parameters were analysed as in section 5.3.2 At test point only the concentration of nitrite, nitrate, BOD_5 and faecal coliform numbers were assessed. Monitoring of wastewater pH, temperature and DO was carried out at intervals through the monitoring period. Spectrophotometric determination of the concentrations of NO_3-N, NO_2-N as elaborated in section 4.3.7.1.5 and 4.3.7.1.6, as well as culture and enumeration of FC and *E. coli* as elaborated in section 4.3.7.2.1 was carried out at 0, 2, 4 and 6 hrs after addition of chemicals. Figure 6.3, 6.4, 6.5 and 6.5 shows changes in parameter in reactor A, B, C and D respectively. Six biological replicates of fresh sample from Hatton wastewater treatment plant were analysed.

6.3 Results and Discussion

The results are summarised in the figures below. Test point was taken at second day (figure 6.2) This test point was determining by assessing the average dissolve oxygen concentration of the experiments in chapter 5 with which a graph of average

DO concentration through the whole treatment time of 11 days was obtained (figure 6.1).

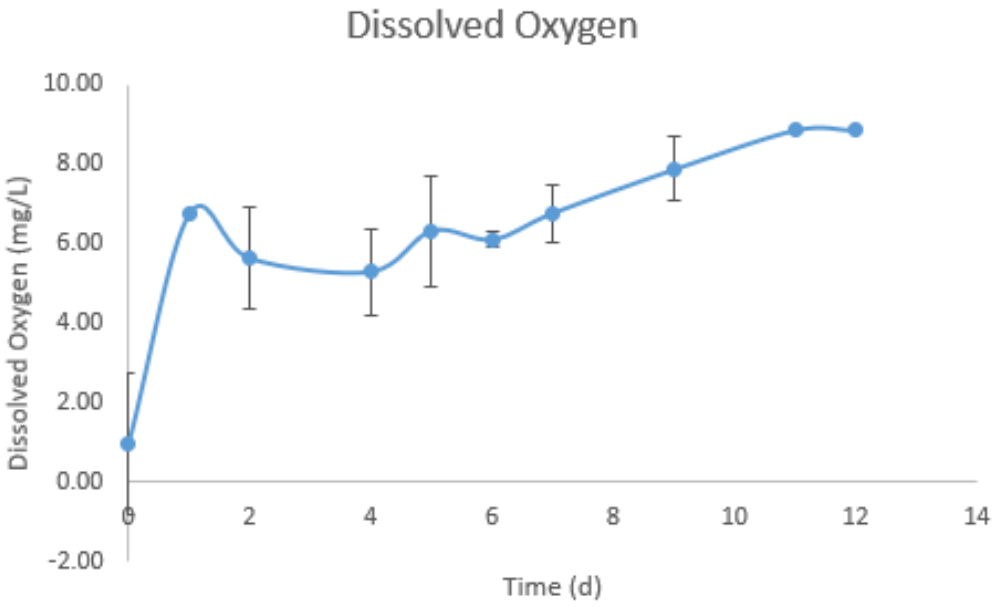


Figure 6-1 Change in dissolve oxygen concentration with time in reactor A

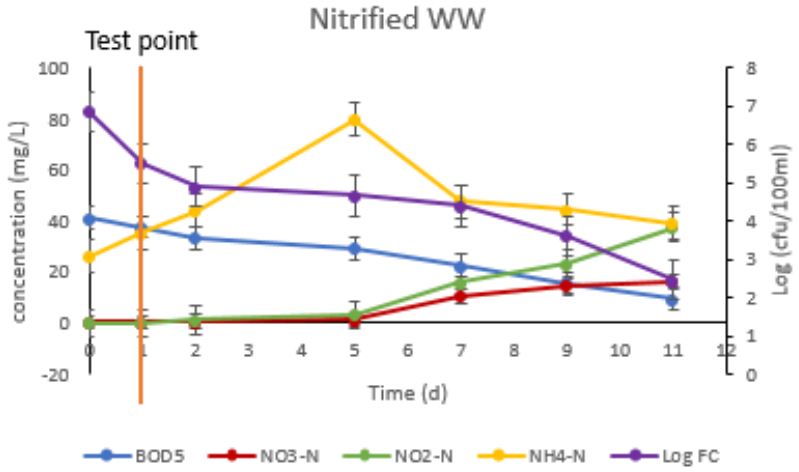


Figure 6-2 Changes in wastewater characteristics as nitrification takes place in A

After 24 hours the average temperature, pH and DO 19.65-20.6°C, 7.99 – 8.03 and DO 5.6 -6.1 mg/L respectively for all reactors These values fall within range

expected for carbonaceous matter oxidation expected at this stage (as was observed in section 5.3.1) and therefore will not be limiting factors here.

Wastewater DO saturation point was taken at day 1 (after 24hrs, figure 6.1) because by this time waste DO concentration was at its peak but it decreased thereafter as oxidation processes proceeded. The DO saturation point was assumed at this point which therefore was test point. This ensured that sufficient oxygen was available in system to allow for possible oxidation of both carbonaceous matter and nitrogenous compound at that point Coskuner and Jassim 2008; Burton *et al.* 2014) in all reactors.

6.3.1 Effects of addition of Allylthiourea (ATU)

Allylthiourea was added to reactors B and C and no increase in nitrogenous oxide quantities were observed in these reactors through the monitoring period though reactor B had a high concentration of the nitrate and nitrite due to the addition of sodium compounds (figure 6.4II). In reactor B however, FC concentration showed a slight decrease but no decrease in FC concentration was observed in C (figure 6.4III). This implied that addition of allylthiourea did not affect concentration of FC in both reactors and confirmed the observation by Bedard and Knowles (1989) who noted that allylthiourea did not affect the activities of other microorganisms in systems in which it was found. Change in FC concentration in B could possibly be attributed to the potential effects of the added nitrite and nitrate.

Changes in FC concentration in A and D will not have been affected by allylthiourea as it was not added to their systems.

Judging by section 5.3.2 nitrogenous oxidation should not occur at this stage due to heterotrophic activity occurring as a result of presence of a lot of carbonaceous matter. However, with no allylthiourea in A the few nitrifiers already present in mixed liquor might carry on limited nitrogenous oxidation but in B and C their activity was hindered by the presence of the inhibitor hence the quantities of nitrates and nitrites observed in the reactors.

Though allylthiourea prevented the oxidation of inorganic nitrogen (Surmacz *et al.* 1996) hence no increase in nitrite, no increase in nitrate was also observed in reactors where it was added. Even in reactor A where no addition of chemicals occurred, its concentration did not increase. Nitrifying bacteria are more sensitive to

change of environment and are slow growers (Mara 2003). It is therefore possible that as test point was close to the beginning of treatment, the nitrifiers in mixed liquor where not fully acclimatised to environment and hence no increase in nitrate concentration was observed.

6.3.2 Effects of addition of nitrate and nitrite

As soluble compounds, sodium nitrate and sodium nitrite dissolve in wastewater resulting in dissociation of the compounds into their ions (equations 6.1 and 6.2) so that an addition of nitrate and nitrite ions respectively leads to initial increase in concentration of nitrate nitrogen and nitrite nitrogen in mixture (figure 6.3III as observed in reactor B and D.

$$NaNO_3(s) \rightarrow Na^+(aq) + NO_3^-(aq) \quad (6.1)$$

$$NaNO_2(aq) \rightarrow Na^+(aq) + NO_2^+(aq) \quad (6.2)$$

Total value of NO_3-N and NO_2-N gave the value of nitrogenous oxides (NO). Average NO concentrations in reactors B and D ranged from 9.06 to 9.86 and nitrite concentrations from 1.02 to 1.47 mg/L after addition of sodium nitrate and sodium nitrite respectively in both reactors B and D.

The quantities of FC in reactors A and C where no addition of nitrate or nitrite occurred, were minimal at range of 5.5 to 5.4 log in both reactors at the end of monitoring period. The minimal reduction of FC was possibly as a result of nitrogenous oxides already present in mixed liquor. More so, the test point occurred at the section of treatment system where heterotrophic activity out competed by autotrophic activity so that organic carbon reduction would be predominant to nitrification (figure 5.6). This is possibly the reason for limited availability of nitrogenous oxides and as well, possibly the reason for very little reduction in FC concentration. Any reduction in FC concentration here would possibly be due to organisms not fully acclimatized to new environment (Mara 2003) as this is just the beginning of treatment process. The availability of organic carbon which is food for heterotrophs implies that conditions of living and growth are beneficial to availability of heterotrophs of which are FC hence less reduction.

In reactor B and D where there was addition of nitrate and nitrite FC concentration reduction was higher than in reactor A and C. Change in quantities of FC ranged

from 5.5 to 4.1 log in both reactors averagely. In B however, the addition of nitrate and nitrite resulted in greater decrease in FC numbers, 4.1 log as oppose to 4.5 log in D. This indicated that the presence of nitrogenous oxides might have an effect on the quantities of FC in both reactors but much more in B. Nitrite concentrations were higher in B than in D at 1.1 and 1.5 respectively This reduction could therefore be as a result of the toxic effect of nitrite (Meays 2004) on FC in these reactors. Conversely in A and C nitrite concentrations were recorded within the range of 0.3 to 0.4 mg/L which below the toxic limit. 1 mg/L nitrite is not toxic to aquatic organisms (Meays 2009) hence its inability to affect the biota in A and C.

It is also observed that the addition of nitrite and nitrate was done at early stages of treatment when ammonification was still occurring so that the presence of amino acids were still present in system and ammonification was ongoing (section 5.3.1) These might have contributed to increase in nitrite hence toxicity as observed in earlier research (Philip and Verstrate (2000).

Another change observed in reactor B and D but not in A and C is the increase in BOD_5 (figure 6.4I). Figure reveals that as FC numbers decreased there is increase in BOD_5. It is possible that death of FC caused by toxicity of nitrite led to increase in organic carbon hence increase in BOD_5. Low concentrations of nitrite in reactor A and C is likely the reason for the absence of BOD_5 increase as toxic impact were not felt by bacteria in those reactors.

Figure 6.4 is a summary of the change in wastewater parameters BOD_5 and nitrogenous oxide (NO) after the addition of salts. The effect of the presence of nitrogenous oxides on FC concentration reveal reactor B and D having greatest reduction in FC possibly as a result of the addition nitrite and nitrate salts.

Paired sample t-test (IBM 2015) revealed statistically significant difference in reduction of FC numbers through test period between reactor A and reactors with no addition of nitrogenous salts. AT 95% confidence level $t=13.5$ $p=0(<0.05)$ and $t=17.1$ $p=0$ (<0.05) when reference reactor was compared with reactors A and D to which the nitrogen salts were added. Conversely $t=1.2$ $p=0.33$ (>0.05) were obtained when A was compared to C to which no salts were added indicating statistically no significant difference in FC concentration when only the inhibitor was added (Table 6.2). This implies that the presence of nitrogenous oxides in the system influences

the number of FC in the system as indicated by the reduction in FC concentration observed.

Table 9 Paire sample t test comparing differenced in FC reduction by modifications of system

Paired Samples Statistics

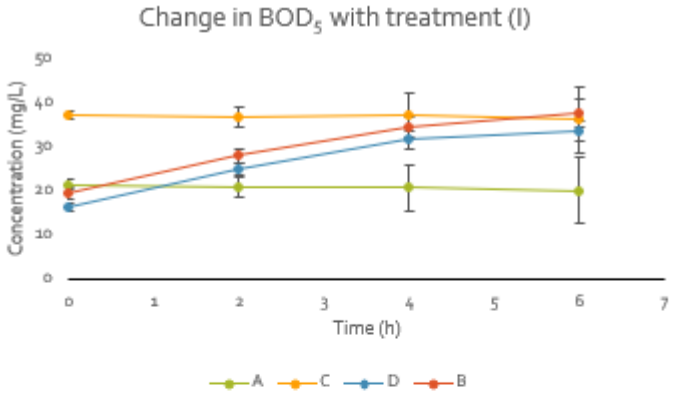
		Mean	N	Std. Deviation	Std. Error Mean
Pair 1	A	5.4000	6	.14142	.05774
	B	4.0667	6	.21602	.08819
Pair 2	A	5.4000	6	.14142	.05774
	C	5.2667	6	.16330	.06667
Pair 3	A	5.4000	6	.14142	.05774
	D	4.1267	6	.15578	.06360

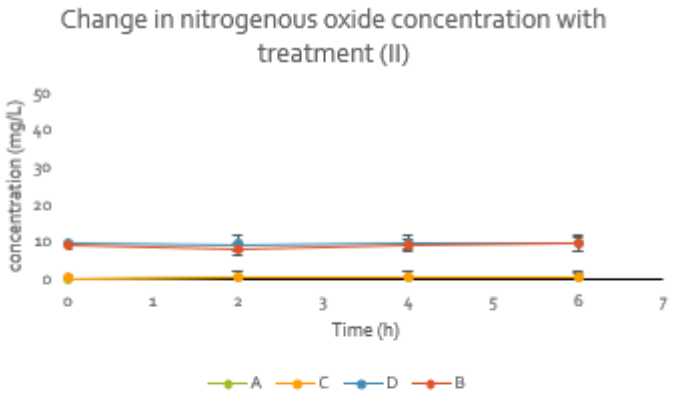
Paired Samples Correlations

		N	Correlation	Sig.
Pair 1	A & B	6	.131	.805
Pair 2	A & C	6	-.953	.003
Pair 3	A & D	6	.254	.627

Paired Samples Test

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference Lower	Upper			
Pair 1	A - B	1.33333	.24221	.09888	1.07915	1.58752	13.484	5	.000
Pair 2	A - C	.13333	.30111	.12293	-.18266	.44933	1.085	5	.328
Pair 3	A - D	1.27333	.18184	.07424	1.08250	1.46417	17.152	5	.000





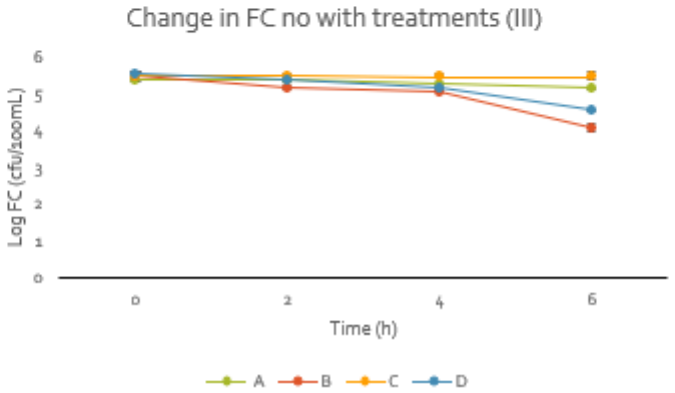


Figure 6-3: Change in wastewater characteristics and FC numbers with different treatments. A: Reference no addition, B: Addition of nitrate and nitrite and allythiourea, C: Addition of allythiourea only, D: Addition of nitrate and nitrate only. Error bars show standard deviation, n=6.

6.4 Key points and Conclusion

The addition of nitrate and nitrite led to a reduction of FC numbers in the bioreactors during monitoring period. Though reduction in FC numbers was observed generally throughout the monitoring period in all reactors, more reduction was observed in reactor with added nitrate and nitrite. This implied that the presence of nitrogenous oxides might be responsible for reduction of FC in this system.

However, an increase in organic matter in the system during this process indicates that other microorganism might have been affected by the presence of the nitrogenous oxides as well and therefore an assessment of system function is important to understand the extent of the effect of the toxicity of nitrite on system function and to minimise the effect of nitrite on other wastewater microorganisms. Moreso, nitrate and nitrite were added together in same reactor so that understanding the individual effect of either is impossible. This is recommended for future investigation on the system.

Also, biota in aquatic systems are known to be preyed on by protozoa (Madoni 2009). It would be necessary to evaluate the impact of protozoa activity on the reduction of microorganism in this system to understand their contribution to the reduction of FC as well.

It is possibly that molecular studies to evaluate the changes in specific faecal bacteria concentration and type with the use of FISH probes in combination with considerations of oxygen utilisation rates (Kim *et al* 2001) as well as any methods targeting ribosomal RNA of FC in nitrifying communities in treatment processes (Mertoglu 2008) will be necessary to provide greater understanding of the changes to microbial diversity and quantity occurring in these systems.

Chapter 7. Effect of Protozoa Predation on Faecal Coliform reduction in Batch Activated Sludge systems

7.1 Introduction

Protozoa are the main predators in WWTS where they prey mostly on suspended bacteria so that their presence has been shown to result in good effluent quality (Madoni 2009). This main activity therefore affects the concentration of microorganism in biological treatment system thereby having a direct influence on the treatment process itself (Stoenicia *et al.* 2014). Indirectly, they are also involved in reduction of organic carbon as they excrete mineral nutrients which increase the utilization of organic carbon by bacteria as well as bacterial growth stimulating substances (Pauli *et al.* 2002). In AS systems, ciliates are the dominant phyla (Curds 1982) and are indicators of treatment performance as their density and diversity has been corelated to treatment plant performance (Madoni 1994). They have been observed to contribute to the reduction of FC in waste stabilisation ponds (Awuah and Gyasi 2014) and the reduction of *E. coli* numbers in (Epinosa-Gracia *et al.* 2014) in oxidation ditches.

Chapters 5 and 6 indicate a possible cause of reduction in faecal coliform numbers in activated sludge systems to be nitrogenous oxidation processes and the presence of nitrogenous oxides respectively, in aerobic AS treatment system. However, understanding pathogen reduction will not be complete without the assessment of the impact to this system of major predators of bacteria in the aquatic systems. Assessments of the influence of protozoa on AS treatment processes can provide additional information on the impacts of pollutants on biological mechanisms therein, as well as indicating longitudinal changes in wastewater contents (Madoni 1994; Pauli and Pauli 2014) through changes in type and quantity of protozoa or by assessing effects of their absence during WWT processes (Pogue and Gibridge 2007).

The effects of predation on wastewater treatment systems have been assessed by the use of substances that inhibit or reduce their presence as this has been observed to alter biotic activities (Papadimitriu *et al.* 2010). Several inhibitory substances like metals e.g. copper, cadmium, zinc, chromium, iron (Nicolau *et al.* 1999, Martine-Gonzalez *et al.* 2006), salts e.g. sodium chloride, chemical

disinfectants e.g. chlorine dioxide (Papadimitriu *et al.* 2007; Rehmah 2008), cyanide, triton X-100 (Nicolau *et al.* 1999) and cycloheximide (Bomo *et al.* 2004; Chabaud *et al.* 2006) have been used. Cycloheximide, used in this study, has been observed to have the advantage of inhibiting growth of protozoa but not causing death of bacteria which all of the other inhibitors do at varying concentrations (Pogue and Gilbride 2007).

Therefore, in a bid to substantiate the information on reduction of faecal coliforms in activated sludge systems obtained in chapters five and six the objective of this chapter is to assess the effects of protozoa presence on faecal coliforms reduction in the present system.

7.2 Methodology

To assess the effects of protozoa on nitrification and pathogen reduction, protozoa inhibition in the batched aerated AS system treating municipal wastewater was investigated in line with previous study by Pogue and Gilbride (2007). The experimental setup was based on the initial treatment setup elaborated in section 4.3. The effects of protozoa inhibition on treatment were assessed by modifying the basic treatment setup established in chapter 5 with the addition of different chemicals as elaborated in table 7-1 below.

Table 10: Experimental methodology

Treatment	*Reactor*			
	A-Reference reactor- nitrification and protozoan activity (n=6)	**ATU no nitrification (n=6)**	**CLY no protozoan activity (n=6)**	**ATU+CLY no nitrification & no protozoan activity (n=6)**
Extended aeration	Yes	Yes	Yes	Yes
Allylthiourea	No	Yes	No	Yes
Cycloheximide	No	No	Yes	Yes

Reactor N was the reference reactor and an exact replica of basic treatment set up established in section 5.3. In reactor ATU, the contents of N were modified by the

addition of allylthiourea (ATU) to inhibit nitrogenous oxidation. To ensure spontaneous action (Bedard and Knowles 1989), a concentration of 3.33 mL/L (2 g/L solution), ATU (Fisher Scientific, UK) was used as in section 6.2.1 to successfully inhibit ammonia oxidation. As oppose to the experiment in chapter 6, its effect on the whole treatment period (11 days as in chapter 5) is assessed in a bid to understand the overall change in faecal coliform numbers in the system without the influence of nitrification.

In reactor CLY the contents of N are modified by the addition of protozoa inhibitor, cycloheximide (CHM) (Fisher scientific, UK), at concentration 200 mg/L (Chabaud *et al.* 2006). CHM is an antibiotic which inhibits 80s ribosomal protein synthesis by interfering with the translocation of protein molecules of eukaryotic organisms thus inhibiting their growth and multiplication (Taylor and Pace 1987). However, it was observed that its inhibitory activity could be partial on protozoan ciliates (Davis *et al.* 1995). CHM inhibition activity was said to require a minimum action of 3 days as significant action has been observed only after 3 days of contact with at least 200 mg/L (Chabaud *et al.* 2006). Also, its action was not observed to affect bacteria die off (Bomo *et al.* 2004).

Lastly, reactor ATU+CLY contents were a modification of N by the addition of 3.33 mL/L ATU and 200 mg/L CHM so that both processes of nitrification and protozoa predation were inhibited through treatment (Pogue and Gilbridge 2007). This was aimed at assessing the change in faecal coliform numbers independent of both processes through treatment.

These four reactors ran in parallel each time with wastewater working volume of 3L and chemicals were added to wastewater immediately before commencement of aeration. Grab samples were collected from the reactors on days 0, 2, 5, 7, 9 and 11, filtered and analysed for physico-chemical parameters temperature (T°C), pH and DO to ensure that the system was always within permitted range for nitrification activity. Also, wastewater parameters NH_4^+-N, NO_2^--N, NO_3^--N, BOD_5, COD and FC numbers were evaluated as elaborated in section 4.3.7 and the experiment was carried out six times.

Supplementary data obtained from an investigation on change in protozoa diversity quantity as a result of the changes occurring in reactor CLY (Duruifeako 2017) was

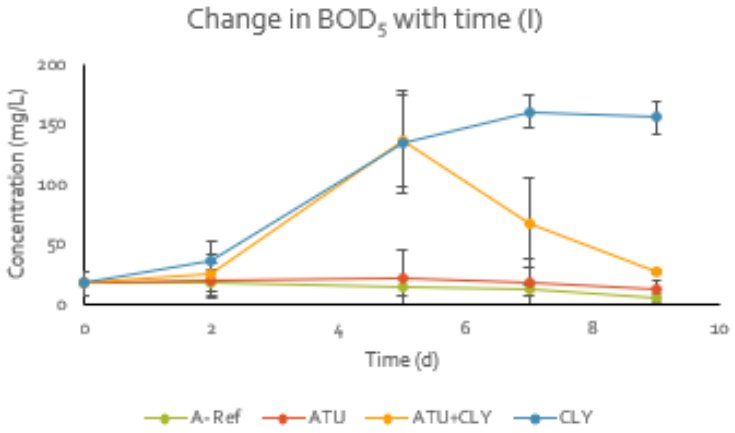
assessed and comparatively analysed with change in concentration of NO_3^--N and FC numbers occurring in CLY to compare the effect of predation to the effect of nitrification on FC numbers.

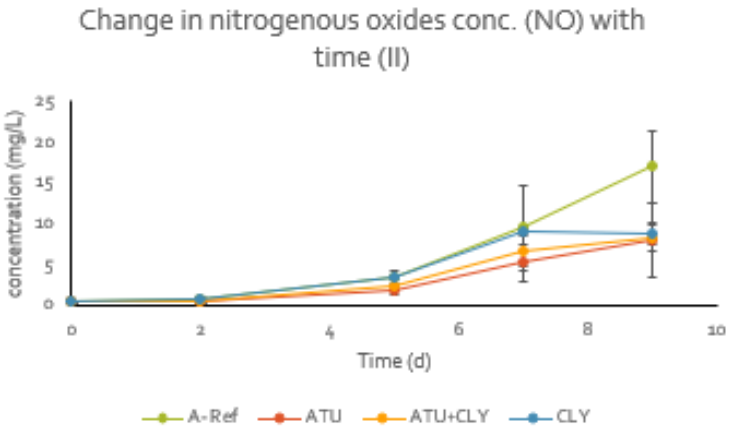
7.3 Results and Discussion

Figures 7-1(I-IV) to 7-4 show average changes in wastewater concentrations of NH_4^+-N, nitrogenous oxides (addition of nitrite and nitrate oxide concentrations), BOD_5 and faecal coliform numbers as treatment progressed simultaneously in all four reactors.

Temperatures monitored ranged from 18.25 to19.75°C in all four reactors. DO levels increased progressively in all the reactors from 3.93 mg/L to 8.85 mg/L averagely because aeration was continuous. For pH, values observed ranged from 6.23 to 8.50 though trend of change in values differed in each reactor. These values of temperature DO and pH respectively were within and above the levels required for nitrification activity (Blackall and Burrell 1999; Chen *et al.* 2006; Coskuner and Jassim 2008). The addition of chemicals did not have an impact on temperature or dissolved oxygen concentration but with pH its value seemed to be affected by the presence of ATU in reactor ATU.

The effect modifications on BOD_5, nitrogenous oxides, ammonium N concentrations and FC numbers were represented graphically in fig 7.1 I. II, III and IV respectively. Changes in the wastewater character in reference reactor (A-Ref) show identical trends to results obtained in section 5.3.


Change in BOD_5 with time (I)
Concentration (mg/L)
200
150
100
50
0
0
2
4
6
8
10
Time (d)
A-Ref
ATU
ATU+CLY
CLY


Change in nitrogenous oxides conc. (NO) with time (II)
concentration (mg/L)
25
20
15
10
5
0
0
2
4
6
8
10
Time (d)
A-Ref
ATU
ATU+CLY
CLY

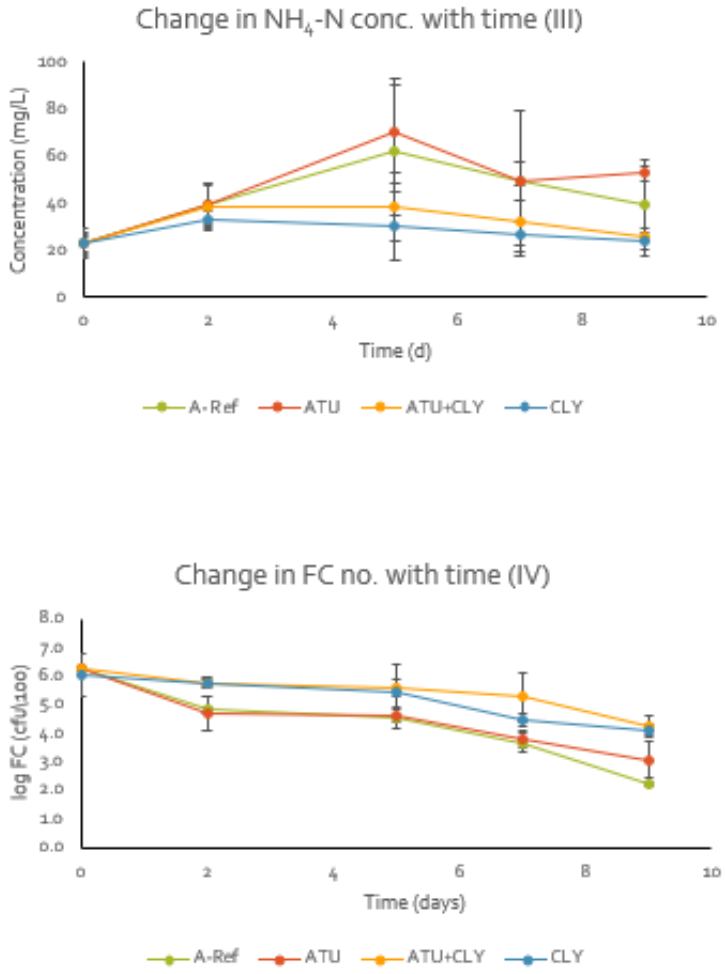


Figure 7-1: Changes in wastewater constituents and FC no. with different modifications. A-Ref: No modification, ATU: Nitrification inhibitition with Allythiourea only, ATU+CLY nitrification and protozoa inhibition with allythiourea and cycloheximide, CLY protozoa inhibition only. Error bars show standard deviation and n = 6

As aeration proceeds in A.Ref a gradual decrease in BOD_5 from 18 mg/L to 11.6 mg/L corresponded to a reduction in FC numbers but FC numbers continue to decrease even after levelling off of BOD_5. Increase in NO occurring concurrently with further FC reduction indicates that reduction could be attributed to the presence of the products of nitrogenous oxidation as in section 5.3.

More NO values were observed in the reference reactors averagely than in the other (fig 7.1 (II)) reactors. NO values for A-ref increased from 0.38 mg/L to 16 mg/L while in reactor ATU, CLY or CLY+ATU it ranged from 0.31 mg/L to 8.31 mg/L only. This implied that nitrification was reduced through the treatment period in reactors with added chemicals.

7.3.1 Effects of inhibition of Nitrification

In reactor ATU where ATU was added changes in concentrations of NO_3, NH_4^+-N, BOD_5 and FC were attributed to inhibition of nitrification through treatment (figure 7.1 I-IV). Change in FC numbers was observed to show an identical pattern to that observed in A-Ref; decrease during period of carbonaceous matter reduction and further decrease during period of nitrogenous oxidation (Figure 7.1IV). In this reactor, nitrification, indicated by rise in NO concentration, was delayed till after day 5 as oppose to day 4 for reference reactor. FC reduction was observed to occur concurrently with increase in nitrogenous oxide concentration (Figure 7.1 II & IV) hence substantiating the effect of nitrogenous oxides presence on FC quantity reduction established in section 5.3. At end of treatment, average FC colony numbers were 3 log at ATU as oppose to 2 log at A-Ref (figure 7.1 IV) indicating that though there was reduction of FC numbers in both reactors this reduction was higher in a situation when nitrification was not inhibited.

The inhibition of ammonia monooxygenase activity by ATU prevents ammonia oxidation so that NH_4^+-N accumulates in the system. This process combined with NH_4^+-N build up already identified to occur during carbonaceous reduction as a result of ammonification and limited nitrification in the system (Section 5.3) results in a greater amount of NH_4^+-N in A (70.5 mg/L) than in N (62.5 mg/L) averagely. The effect of this was probably felt in reactor ATU by increased pH to 8.5 which was possibly caused by a shift in the equilibrium between NH_4^+-N and free ammonia (FA) moving towards free ammonia as suggested in previous research (Anthonisen *et al.*

(1976); Pogue and Gilbride 2007). This possibly caused a delay and reduction in the occurrence of nitrogenous oxidation in the system and therefore less effect on the reduction of FC numbers in the system as compared reference reactor. However, the reactions in this reactor indicated, that without nitrification, FC numbers will reduce by the action of other processes in the system, but reduction will be less than with nitrification.

The occurrence of nitrification in this reactor after day 5 indicated that ATU inhibition may have been continuous through treatment confirming previous research which stated that allylthiourea's inhibitory activity was not very effective after 5-day period (APHA 2001) and that during its action nitrite oxidisers remain inactive (Ginestat *et al.* 1998) so that after period of effective inhibition nitrification activity resumes. Confirming the observation by Progue and Gilbride (2007) who stated that ATU delays nitrification.

7.3.2 Effects of Protozoa presence

The effects of protozoa inhibitor CHM were used to assess the effects of protozoa activity on system by assessing the concentrations of NH_4^+-N, NO, BOD_5 and FC numbers through treatment time in reactor CLY and its effect on these wastewater characteristic are presented through figure 7.1 I-IV. At IV changes in FC numbers appeared identical to that observed in A-Ref with a gradual decline during the carbonaceous reduction phase and a sharper decrease with respects to nitrogenous oxidation.

The addition of CHM seemed to have caused several changes in wastewater character in this system. Firstly, after the third day, a sharp and consistent rise in BOD_5 till end of treatment was observed (figure I). The addition of CHM may have caused death of protozoa who have been starved by lack of prey so that presence of death, decaying cells would increase organic carbon in the system. This confirmed the observation of Pogue and Gilbride (2007) who observed an increase in BOD_5 in their reactor after addition of CHM.

Secondly NH_4^+-N concentrations (Figure 7.1 III) were lower in CLY (maximum of 38 mg/L) than in A-Ref (maximum of 62 mg/L). Probably the absence of protozoa resulted to an increase in heterotrophic bacteria in the system. This may have led to increased heterotrophic bacteria assimilation of ammonia thereby causing a

reduction in NH_4^+-N concentrations. More so, average NO values observed in this reactor were 8.8 mg/L maximum as opposed to 16.8 mg/L observed in reactor N indicating reduction in nitrification activity which is possibly as a result of reduced ammonia availability. These latter two observations agree with Hanaki *et al*. (1989) who stated that increased heterotrophic growth in suspended growth systems resulted in the process of heterotrophic assimilation of ammonia occurring in preference to nitrification. In addition, was the increased organic matter presence which led to a situation whereby nitrogenous oxidation was inhibited due to autotrophs being out competed for oxygen by hetrotrophs.

The above reasons therefore resulted in reduced nitrification occurrence which therefore caused a relative decrease in reduction in FC numbers in system when compared to reduction in reference reactor (Figure 7.1 IV). This implies therefore that if protozoa were present their predatory activity would control the quantity of heterotrophs in the system, increasing nitrification rate and increasing the reduction of FC.

7.3.3 The effect of protozoa absence in the absence of nitrification

The effects of inhibiting both nitrification activity and predation on changes in wastewater character and FC numbers as occurred in reactor CLY+ATU are represented and account for the effect of protozoa presence on faecal coliform reduction without the influence of nitrogenous oxidation.

FC numbers decreased in identical trend observed in other reactors, but decrease was lower than in reactors with no CHM added (Figure 7-1 IV). This decrease in reduction may be as a result of the changes in other parameters in reactor AC. As with section 7.1 IV BOD_5 increased at day 2 sharply, possibly confirming that the increase in BOD_5 was as a result of addition of CHM. Also, BOD_5 decreased consistently after day 5 till the end of treatment contrary to the continuous increase in reactor Cl (Figure 7.I (I)). It is possible that ATU presence reduced the effect of CHM on protozoa activity so that after day five the effects of death organic matter on system reduced and organic carbon reduction by heterotrophs became noticeable. Once more as NO concentration became prominent (after day 5) further reduction in FC numbers were observed.

It appears the presence of ATU prevented complete inhibition of protozoa activity in this system as oppose to reactor CLY. Average concentrations of NH_4^+-N and NO was 25.85 mg/L and 8.22 mg/L respectively which were identical to that observed in CLY supporting the fact that CHM presence reduces ammonia concentrations and nitrification activity. In this reactor the effects of no protozoa activity were obvious before day 5 with no nitrification, increase in organic carbon as in section 7.2.2 and were identical to section 7.2.1 as nitrification occurred after day 5. The result was higher numbers of FC observed at end of treatment in AtU+CLY than in ATU or CLY. This confirms the suggestion by Petropoulus and Pogue (2005) that changes in process parameters in the inhibition of nitrification could be detrimental to protozoan activity and so modify nitrifying performance of the system. Low nitrifying ability with protozoa inhibition has also been attributed to the reduction of cell growth nutrients secreted into the system by the presence of protozoa (Petropoulus and Pogue 2005; Madoni *et al.* 2009). These therefore suggesting that in the absence of both protozoa and nitrification in AS systems, FC reduction will be low. Possibly, both protozoa presence and nitrification are important in the reduction of FC.

7.3.4 Effects of protozoa inhibition on FC numbers

There was a general reduction in FC numbers in all different system modifications as indicated in figures 7-1 (IV). However, in figure CLY and and ATU FC numbers are higher (4 log) at end of treatment time than in A-Ref and ATU+CLY representing systems with no CHM (2 log and 3 log respectively). In reactor N without modification, the lowest number of FC colonies were enumerated, and AC presented the highest number of FC colonies indicating greatest and least reduction in FC numbers respectively.

These results indicate that protozoa are important in the reduction of FC numbers in these nitrifying systems. At both periods of organic matter reduction and nitrification in all reactors there was some amount of reduction in FC numbers. More so, despite the increases in BOD_5 concentrations indicating availability of large quantities of food (organic carbon) observed in CLY and ATU+CLY, FC numbers still reduce gradually though reduction was little (figure 7.1 I & IV). In these systems therefore, reduction in FC numbers appears to be influenced by other factors than food quantities. Also, it is possible to infer that ATU and CHM seem to have no direct influence on FC numbers, as the trends in reduction are similar in all reactors.

In all reactors it was observed that increase in concentration of NO also coincided with reduction in the FC numbers through treatment period thereby linking the reduction in FC numbers to occurrence of nitrification. Larger number of FC numbers indicated that FC removal was reduced as protozoa activity was inhibited in CLY and ATU+CLY reactors. Possibly inhibition reduced the indiscriminate grazing activity by protozoa on suspended bacteria amongst which are FC as suggested by (Petropoulus and Gilbride 2005, Chadaud *et al.* 2006 and Pogue and Gilbride 2007) thereby leading to reduction in bacteria numbers in AS systems. This suggestion is consistent with previous research by Madoni *et al.* (2009) in which *E. coli* numbers were reduced in the presence of ciliates. Moussa *et al.* (2000) also observed a great increase in biomass due to the absence of protozoa and this increase in biomass depleted the system of ammonia and growth nutrient necessary for nitrifiers (Petropoulos and Gilbride 2005) thereby reducing nitrification activity and FC reduction.

The results of this study are consistent with Salvado (1995) in which full scale plants showed a negative correlation between ciliate and heterotrophic activity, limiting nitrification activity when protozoa were absent. More so, the results are supported by other studies which reveal that protozoa predation is selective of size, viability, abundance and shape of prey but does not consider specie (Harvey *et al.* 2002; Gruber *et al.* 2009) of bacteria so that different bacteria mediated aquatic processes mal function when they are absent.

This effect of protozoa on nitrification is contrary to other investigations aimed at linking the activity of heterotrophic, autotrophic and predatory organisms in AS, where a suppression of predatory activity increased bacteria availability hence nitrification (Moussa *et al.* 2005). However, in another study, protozoa presence was found to have no influence on nitrification (Lee & Oleszkiewicz, 2003; Sherr *et al.* 1988; Verhaghan *et al.* 1995) thereby indicating that the results of this study may not be generalised as protozoa activity appears to vary. Also, though CHM can inhibit most predatory protozoa, ciliates were observed to be only partially inhibited by it sometimes (Davis *et al.* 1995) and possible were active here after some time. Earlier research (Pogue and Gilbride 2007) state that the inhibition of nitrification makes it difficult to determine the effect of predation on a nitrifying system as observed in this

reactor. This implies that the effects of predation appear linked to nitrification as observed in the present study.

On assessing the change in ciliate numbers as treatment progressed in this nitrifying bioreactor, secondary data suggested a decrease in average numbers in ten fields from 507.1 to 0.7 when filtered samples were used (Duruifeako and Cameron, 2017). The secondary information was applied to results of present study and represented in figure 7-6 showing the changes in FC numbers related to protozoa numbers and NO_3-N concentrations during treatment. Higher numbers of protozoa are seen at beginning of treatment than at end therefore implying that their effect on FC quantities takes place at beginning of treatment system when carbonaceous matter reduction was occurring while the effect of NO_3-N on FC reduction seems to occur later. A positive correlation ($r= 0.933$, $p>0.05$) exists between log FC and ciliate numbers (Table 7.1). This implies that the decrease in carbonaceous matter reduces both the quantities of protozoa and FC. Protozoa presence in mixed liquor provide growth nutrients through excretory products for bacteria so that heterotrophic activity would increase thereby reducing available as organic matter. More so, the protozoa consume fast growing heterotrophic bacteria in suspension by phagocytosis so that their presence would influence both organic carbon reduction and bacteria quantities.

These estimates of ciliate quantities were obtained from filtered samples and decrease in ciliate quantities in treatment system occur in response to changing environment of decreasing organic carbon (Pauli *et al.* 2001) moving from crawling in the waterphase to sessile (Madoni 1994) as sludge formation proceeds. Also, increases in NO_3^--N (figure 7.6) correspond to decrease in both log FC and ciliate concentrations suggesting that the process of nitrification could be responsible for reduction in both ciliate and FC numbers. Pearson's correlation (IBM 2015) hereby indicates a significantly negative correlation between NO_3^--N and FC ($r=-0.933$) similar to observation in section 5.3. Also, a negative correlation between NO_3^--N and protozoa numbers ($r=-0.756$) indicates that the toxic effect of nitrite accumulation as a result of the nitrification process affected the quantities of protozoa in the system.

Use of one paired sample t test (SPSS, IBM 2015) at 95% confidence level showed statistical significant difference (Table 7.3) between the reduction of FC numbers in reactor A.Ref and ATU (t= 3.7, p=0.02 <0.05), between A.Ref and ATU+CLY (t=17, p=0) and between A.Ref and CLY (t=14.7, p=0<0.05) respectively. This indicated that all modifications reduced the decrease in FC numbers indicating therefore that both processes are relevant in pathogen reduction.

Table 11 Correlation between factors responsible for FC reduction

Correlations

		NO3N	logFC	Protozoa
NO3N	Pearson Correlation	1	-.933*	-.756
	Sig. (2-tailed)		.021	.139
	N	5	5	5
logFC	Pearson Correlation	-.933*	1	.802
	Sig. (2-tailed)	.021		.102
	N	5	5	5
Protozoa	Pearson Correlation	-.756	.802	1
	Sig. (2-tailed)	.139	.102	
	N	5	5	5

*. Correlation is significant at the 0.05 level (2-tailed).

Table 12 Paired sample test showing difference in FC reduction due to treatment

Paired Samples Test

		Paired Differences					t	df	Sig. (2-tailed)
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference Lower	Upper			
Pair 1	R.ref - ATU	-.68400	.41675	.18638	-1.20146	-.16654	-3.670	4	.021
Pair 2	R.ref - AC	-1.96800	.25946	.11603	-2.29016	-1.64584	-16.960	4	.000
Pair 3	R.ref - CLY	-2.21200	.33641	.15045	-2.62971	-1.79429	-14.703	4	.000

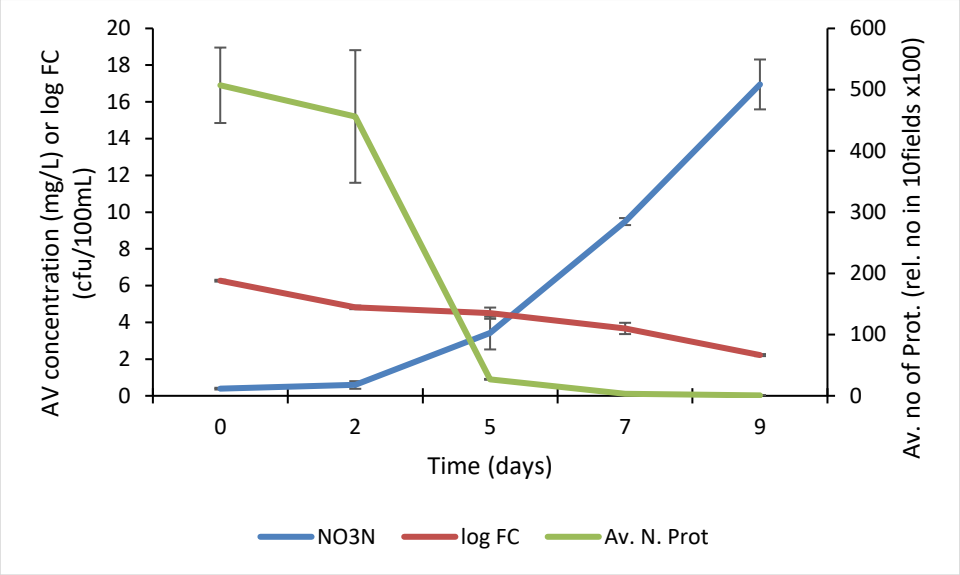


Figure 7-2 Change in FC numbers as a result of nitrification and protozoa presence (Error bar indicate standard deviation)

7.4 Key points and Conclusion

The addition of ATU and CHM were effective in inhibiting both nitrification and protozoa predation respectively, in the system and their presence resulted in a decrease in the reduction of FC numbers in the system. This implied that both nitrification and protozoa were relevant in the reduction of FC numbers in the system.

The effect of protozoa on FC appeared to occur at the initial part of treatment process during carbonaceous matter reduction where protozoa numbers were highest while the effect of nitrification was predominant at the latter part of treatment where nitrogenous oxides occurred in high quantities.

In any case, for substantial reduction in FC numbers both protozoa presence and nitrification appear important. The assessment of total biomass may be required to confirm this assertion as an increase in total biomass concentration was positively correlated with ciliate biomass (Madoni 1994).

Chapter 8. Discussion and Conclusion

8.1 Key outcomes and discussion

This research was conducted to assess the potential of biological wastewater treatment systems in contributing to disinfection of municipal wastewater. The research focuses on the microbial mediated processes occurring at secondary treatment and their effect on faecal coliform and *E. coli* numbers. The activated sludge system which is the most common biological treatment system was the case study. The focus was the occurrence of the process of nitrification and pathogen reduction at secondary treatment during the treatment of municipal wastewater in which nitrogen and pathogens were important pollutants of domestic source. Specifically, as both pollutants were always available in municipal wastewater sources, the research was seeking to assess any link between both contaminants or the effects they could have on each other.

Three main studies were involved in this research project. Firstly, experiments were carried out to establish the possible scale of nitrification pertinent to this wastewater sample in the laboratory activated sludge reactor to establish the factors responsible for nitrification as well as the extent of reduction of nitrogenous and pathogenic contaminants. Also, the identification of persistent pathogens was carried out.

In the second study an assessment of the effect of nitrogenous oxidation on faecal coliforms numbers was conducted by evaluating the direct effect of the presence of nitrogenous oxides on faecal coliform numbers quantitatively as change in FC numbers were enumerated by spread plate colony counts after the addition of sodium nitrate and sodium nitrite into the system. The third study was designed to confirm the effect of nitrification by assessing the contribution of protozoa predation on FC in the bioreactor during treatment. This was carried out by assessing the impact of inhibition of predation of protozoa on both carbonaceous matter oxidation and nitrogenous oxidation as well as assessing the change in FC numbers through the treatment process.

In the first study nitrification was assessed by observing changes in the quantities of physico – chemical parameters, BOD_5, NO_3^--N and NH_4^+-N and pathogen reduction was assessed by enumeration of FC and *E. coli* colonies grown on HiCrom agar through treatment. Overall, average decreases in organic matter and ammonia,

increase in nitrate concentrations and decrease in *E. coli* and FC numbers were observed in this system as treatment progressed longitudinally. Sufficient organic matter reduction occurred during a period of 4 days thereby permitting the occurrence of nitrogenous oxidation. Aerating and stirring the wastewater continuously for 11 days were important factors in keeping both carbonaceous removal and nitrification on going in the system as DO, pH and temperature were within range required for nitrification activity. During the period of organic matter reduction, increases in inorganic nitrogen concentration were observed and this was attributed to the continuation of ammonification of organic nitrogen and no nitrogenous oxidation at this stage. However, with the presence of excess amounts of DO, reduction of organic matter was subsequently followed by oxidation of inorganic nitrogen till about 11 days.

Reduction of average FC numbers from 6.5 log to 4.5 log was observed during organic carbon reduction and thereafter further down to 2 log average when nitrogenous oxidation occurred. FC reduction at organic matter reduction stage was attributed to longitudinal reduction in food and attachment to sludge floc forming. While reduction thereafter was concurrent with increases in the concentrations of NO_3^--N and NO_2^--N. However, causes of reduction at nitrogenous oxidation stage were unclear and prompted investigation on the effect of the presence of nitrogenous oxides and the impact of predation on the reduction of FC in this system in the next studies.

Also, API 20E identification of persistent FC revealed the presence of *E. coli* and FC including *Salmonella, Klebsiella, Enterobacter, Pseudomonas, Citrobacter* and *Chromo* spp. Noticeable is the dual importance of some as pathogens were involved in either organic carbon reduction or nitrogenous oxidation e.g. *Enterobacter sakazaki* involved in organic nitrogen reduction and *Klebsiella* spp. which indicate an overly aerated environment and occurrence of nitrification. As concentration of substrates for these processes reduce with time, reduction in quantities of these organisms is eminent as starvation leading to death will reduce the number of viable cells. This implies therefore that progress of these processes appear to have an impact in the reduction in quantities of these organisms in the system.

Though the reduction of nitrogen and pathogens independently is well documented in literature, this study is the first step to indicate a link between both processes hence the possibility of concurrent elimination of both pollutants. This stage identified a significantly negative correlation between nitrification and FC in this system however the reason for this relationship was not clear hence the next study.

In the subsequent study, the effects of the products of nitrification on faecal coliform and *E. coli* numbers was assessed. Nitrogenous oxides obtained from the introduction and dissociation of soluble sodium nitrite and nitrate salts, in the system, were seen to reduce the number of FC and cause an increase in organic carbon as indicated by increase in BOD_5 concentration during monitored period. It confirmed the assertion from the previous study of an inverse relationship between the presence of nitrogenous oxides and FC thereby substantiating that FC presence was adversely affected by their presence. These results agreed with Philip and Verstrate (2000) as apparently, the toxic effect of nitrifying species (NH_3, NO_2^-) in the biological systems caused death of microorganism including FC and *E. coli* indicating that at stages of treatment where these species are available pathogen reduction in eminent. The presence of nitrite and free ammonia in aerobic systems have been well documented (Ciudad *et al.* 2006; Hellinga *et al*.1998; Kuai and Verstrate 1998; Zhou *et al.* 2011) and research indicates that advance treatment makes use of them as they have resulted in effective resource management like the lowering of oxygen demand for nitrification, reduction in size of reactors due to HRT reduction, small sludge production and higher denitrification rates (Zhou *et al.* 2011). This study suggests the benefit of pathogen reduction with the presence of these toxic specie which in the system but confirmation on this assertion was required as research (Madoni 1994) already indicated a great cause of reduction of bacteria quantities as protozoa predation

The third study of this research investigated the impact of the presence of protozoa, previously identified as major predators of microorganisms in BWWTS (Pauli *et al.* 2001), on FC numbers in the present system. The effects of predation were evaluated by assessing change in FC numbers when protozoan activity and nitrification in the system were inhibited. This was done by the addition of protozoan and nitrification inhibitors into system at different configurations before treatment. Results indicated that inhibition of either protozoa activity or the nitrification process

independently or together led to increase in the number of viable FC colonies observed on culture media hence less reduction in FC numbers in the system at the end of treatment. Inhibition of protozoan predation possibly allowed for overgrowth of all types hence the availability of more FC at end of treatment. Also, more heterotrophs in the system implied not only limited oxygen for nitrifiers due to competitive advantage but use up of ammonia for growth (Petropoulos and Gilbride 2005) so that nitrification was reduced due to limited substrate. It was observed in these systems with reduced nitrification evidence, that there was less reduction in FC numbers. The results implied that protozoa presence affected FC numbers by direct predation of suspended bacteria of which some FC are involved and indirectly by reducing nitrification activity.

More analysis with secondary data obtained from present treatment system, revealed a greater abundance of ciliates at beginning of treatment at time of organic carbon reduction and a reduction during nitrogenous oxidation stage. This therefore indicated that predation contributed to FC reduction at earlier stages of treatment and nitrification was important at latter stages. Though the effect of predation on nitrification had been previously investigated (Pogue and Gilbride 2007, Petropoulos and Gilbride 2005), its indirect effect on FC by affecting nitrification activity was not previously elaborated.

The changes in this present system as a result of the occurrence of the process of nitrification therefore appear to affect faecal coliform numbers and other processes therein as a result of the presence of nitrite at later stages. Previous research (Phillips *et al.* 2002) state that the presence of nitrite in WWTS systems has been observed to inhibit biological processes by decreasing metabolic activities and growth as a result of hindrance to activity of ATPase in cells thereby leading to bioreactor failure. This makes use of nitrite a critical situation and the observation on nitrite effect on protozoa and the fact that nitrification was not optimal in this system seemingly support criticism. However, the use of nitrite presence in advance biological systems like ANNAMOX indicates that some biological processes are still possible in the presence of nitrite. This thereby implies that the presence of nitrite does not necessary lead to reactor failure. More so, nitrite action has been observed to be reversible (Strous *et al.* 1999; Lotti *et al*. 2012) or irreversible (Jetten *et al.* 2005) in biological system so that it is possible that some adverse effect of nitrite on

biological processes reversed. Therefore, it will be recommended to do more test to assess the nature of nitrite action in this system so as to assess the extent of exposure of organisms to nitrite which could be accommodated by system with limited detrimental effect to biological system.

The applicability of this study would mean that failure of nitrification would limit pathogen reduction of secondary effluent, but also complete nitrification will limit pathogen reduction as well. The failure of nitrification has been observed in full scale WWTS due to the variability in growth and sensitivity of nitrifying bacteria (Cydzik-Kwiatkowska and Zielinska 2016; Wagner and Loy 2002). Possibly bio augmentation with acclimatized nitrifiers may be implemented to limit the possible effects of insufficient growth of nitrifiers (Tang and Chen 2015) and possibly enhance FC removal.

Different nitrifying systems have reported different ratios of ammonia oxidisers to nitrite oxidisers. A 2:1 ratio is theoretically identified (Mairi *et al.* 2012), 2.2 to 2.7 for sufficient nitrification (Li *et al.* 2007), 3.5 to 1 when municipal wastewater was involved (Harms *et al.* 2003; You *et al.* 2003) such that there is great variety in the presence of both nitrifying bacteria in treatment systems. It is therefore possible that not all nitrifying systems will be capable of experiencing reduction in pathogens as a result of nitrification as ratios of nitrifying bacteria hence nitrogenous oxidation species, vary with respect of treatment system (Yao and Peng 2017) and even specific constituent of wastewater at time (Kumari *et al*. 2011).

Bulking would cause increase in pathogens concentration at secondary treatment and the presence of nitrite in WWTS has been linked to filamentous bulking as a result of poor settling properties of sludge (Ma *et al.* 2009; Musvoto *et al.* 1999) but this is not always the case as good settling was observed in nitrifying systems containing 10-15 mg/L of nitrite (Ma *et al.* 2013). This supports the point that nitrite presence, which is necessary for FC reduction, does not always result in failure of system. However, even previous research expresses a need for investigations to assess operation strategies that will support this (Ge *et al*. 2015) and this will be viable system for pathogen reduction.

8.2 General Conclusion

We hypothesize that extensively aerated laboratory scale nitrifying wastewater treatment systems are disinfecting system due to the occurrence of carbonaceous matter oxidation, protozoa predation, floc formation and nitrogenous oxidation processed occurring therein. In the assessment of the inverse relationship between nitrification and pathogen reduction, the presence of free ammonia and nitrogen oxides appears to be the factor causing the changes occurring therein. Though unwanted in aquatic biological systems nitrite presence in municipal systems will enhance pathogen removal.

This work has highlighted the importance of inorganic nitrogen compounds in limiting the presence of pathogens in WWTS. It has explored the possibility of pathogen reduction being induced by natural causes due to on-going biological processes as no addition of chemicals is required. The emergence of new bacteria pathogens resistant to antibiotic and other chemical treatment highlights the need for more 'natural' pathogen reduction processes. It highlights also the usefulness of secondary treatment in contributing to disinfection of wastewater and much more carrying out pathogen reduction and nitrification concurrently.

8.3 Perspectives

The different nitrifying bacteria respond differently to changes in environmental condition thereby affecting their quantities and function in aquatic systems. However, due to their sensitive to environmental changes, different species of ammonia oxidising and nitrite oxidising bacteria could be present in different systems depending on prevalent conditions (Graham *et al.* 2007) so that applicability of study would only be possible if conditions for specific species or conditions of diversity are met. Future work therefore would involve assessing variations in quantity and diversity of nitrifiers through treatment by use of molecular methods, so as to shed more light on the dominant nitrifiers responsible for pathogen reduction in the system.

This work uses the batch activated sludge as a case study and therefore the system design of other secondary treatment systems was not considered thereby limiting its applicability. It will be recommended to assess the possibility and extent of pathogen

removal by nitrification in these systems as well both at laboratory scale and full-scale systems to get a wider picture.

Gethi *et al.* (2018) site the main concern with pathogen reduction as being their ability to regrow in treatment processes. As this investigation was localised at secondary treatment only, it would also be necessary to investigate the possible impact of this pathogen removal process on the subsequent treatment processes in the system and more so its impact on disinfection processes at tertiary treatment stage. Presence of nitrite having been observed to distort chorine disinfection processes (Lotti *et al.* 2012).

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